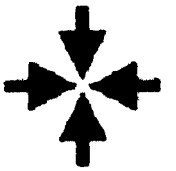
Phenotypical and functional studies on putative cancer stem cells of human melanoma

Thesis



Perego Michela

Degree in Biomolecular Biology

PHENOTYPICAL AND FUNCTIONAL STUDIES ON PUTATIVE CANCER STEM CELLS OF HUMAN MELANOMA

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"Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science."

J. H. Poincaré

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ABSTRACT

Several lines of evidence obtained in a variety of human tumours support the theory of cancer stem cells (CSCs). The aim of this thesis was the isolation and the biomolecular characterization of the tumorigenic subpopulation of melanoma cells.

Melanoma cells growing in stem cell medium as non-adherent colonies, here named 'melanospheres', were successfully isolated from melanoma specimens or from melanoma cell lines. Melanospheres displayed a self-renewal capacity and multipotency *in vitro* and were highly tumorigenic *in vivo*. Remarkably, melanosphere-derived xenografts maintained tumorigenic potential in subsequent recipients and mirrored the original melanoma of the patient.

Melanospheres expressed a heterogeneous assortment of stem cell markers, and no direct and unique correlation between any of their phenotypes and *in vivo* tumorigenicity was found.

Conversely, melanoma cells cultured in the presence of fetal calf serum displayed a lower tumorigenicity in SCID mice with limited engraftment capacity in secondary recipient animals. Moreover, adherent cell xenografts displayed a homogeneous phenotype for the expression of melanoma associated markers and contained cells with markers of full differentiation.

Taken together our data provide further evidence on the heterogeneous nature of human melanomas and show that melanospheres and their xenografted tumours represent a useful model to investigate melanoma biology.

The parent-to-progeny relationship between CSCs and tumour bulk does not necessarily reflect the well conserved and predictable rules operating in normal tissue development. CSCs may thus not be a static compartment but rather

stemness features can be acquired by tumour cells in response to environmental signals. Thus, in this thesis, experiments have been performed to define the role of tumour environmental factors produced by melanoma cells themselves or by cells composing the tumour stroma in modulating the acquisition of stemness properties.

The results provided in this thesis indicated that melanospheres display an extensive secretory capacity quantitatively and qualitatively different from that of melanoma cells growing as adherent monolayer. The roles of these immune-related factors in shaping melanoma heterogeneity, plasticity and tumour maintenance are discussed.

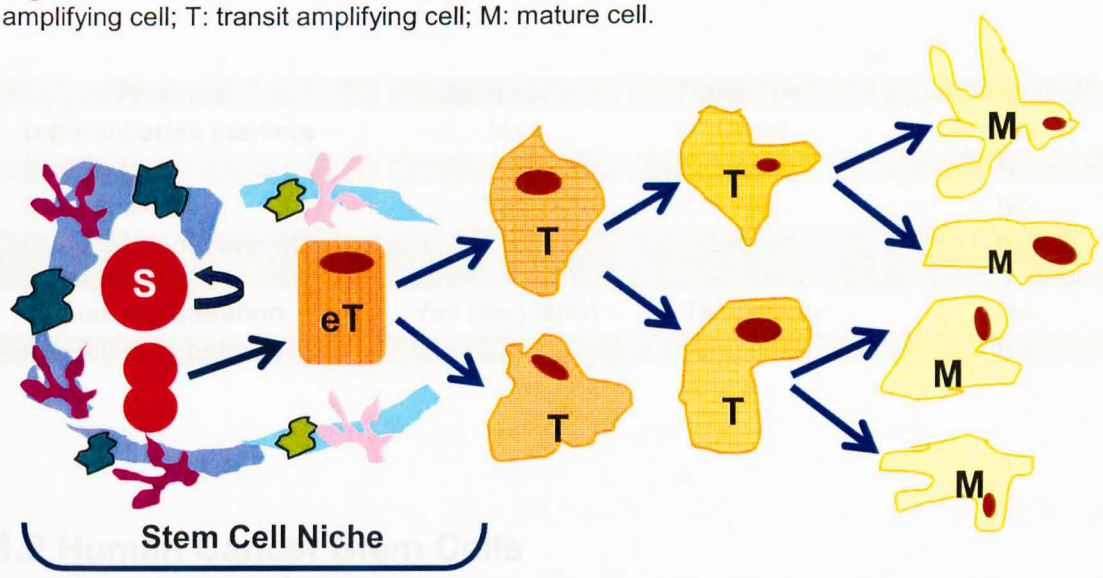
1.1 STEM CELL AND CANCER

1.1.1 Normal adult Stem Cells

Normal adult stem cells (SCs) reside in most somatic tissues, where they are responsible for the maintenance of tissue homeostasis. These SCs are relatively rare and usually they do not express differentiation markers and they do not display other morphological features specific for the mature cells of the tissue they belong to. SCs are instead endowed with the ability to self-renew and to generate transit amplifying progenitors committed to differentiate.

Self-renewal is defined as the ability of stem cells to go through numerous cycles of cell divisions maintaining constant their number for virtually unlimited period of time. Stem cells, through asymmetric division, originate cells that maintain all the features of stemness, and progenitor cells that instead will undergo progressive differentiation thus supporting both tissue and stem cell homeostasis (Reya et al, 2001) (Figure 1.1).

Fig. 1.1. Scheme of stem cell location and progeny. S: Stem cells; eT: early Transit amplifying cell; T: transit amplifying cell; M: mature cell.



In conditions such as a serious tissue damage that affects SC compartment, SCs can divide symmetrically, thus generating cells and recovering the stem cells compartment. Stem cells can also remain in quiescent status and re-start proliferation needed only to maintain tissue homeostasis.

Despite the importance of stem cell self-renewal, we are only beginning to understand how it is regulated. The coordinate activities of multiple pathways are required for a stem cell to self-renew. Wnt, Notch, Hedgehog as well as genes regulating chromatin structure such as Bmi-1 are crucial for the self-renewal of different adult stem cells (Molofsky et al, 2004). The balance between self-renewal and differentiation is maintained by the interplay between these pathways and the differentiation signals received by the stem compartment from cells and other tissue components (cellular matrix, blood vessels), altogether constituting the" stem cell niche" (Morrison and Kimble, 2006). The main characteristics defining stem cells are summarized in Table 1.1.

Table 1.1 Stem cell features. (Adapted from Potten and Loeffler, 1990).

Features	*Stem cells*	*Transit cells*	*Maturing cells*
1. **Differentiation markers**	No	Onset	Yes
2. **Proliferation**	Yes	Yes	No
3. **Self-renewal**	Yes	No	No
4. **Generation of committed progeny**	Yes	Limited	No
5. **Tissue regeneration**	Yes (long term)	Temporarily	No
6. **Flexibility in options**	Yes	Yes	No

1.1.2 Human Cancer Stem Cells

1.1.2.1 The Cancer Stem Cells hypothesis

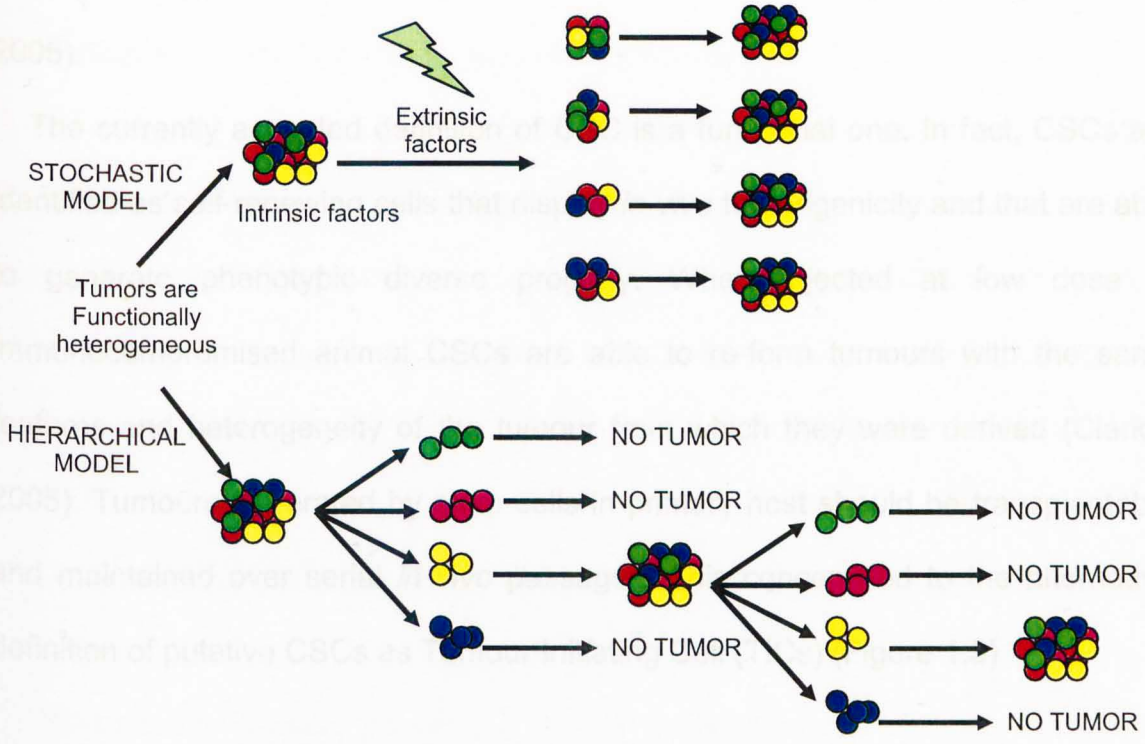
Tumours differ from physiological tissues because they lose homeostatic mechanisms (Leong and Karsan, 2006); moreover tumour cells proliferate extensively, exhibit a heterogeneous phenotype and are composed by cells with varying clonogenic capacity and displaying some degree of plasticity (Hendrix et al., 2003).

Traditionally two models have been proposed to explain tumour heterogeneity: the stochastic (or clonal evolution model) and the hierarchical model. The stochastic model assumes that some mutant tumour cells with a growth advantage are selected and expanded. The stochastic event that governs tumour-initiating capacity can be intrinsic, such as the activation of critical molecular pathways or extrinsic, such as the requirement of the appropriate microenvironment. This indicates that genetically divergent clones of tumorigenic cells can arise and maintain malignant potential independently, also providing a basis for understanding genetic mechanisms of therapy resistance acquired by cancer cells.

The stochastic model states that all cells with the same genetic alteration have equal potential to regenerate the tumour, thus, according to this model, the prospective isolation of tumour initiating cells is unfeasible.

In contrast, the hierarchical model postulates that only a subset of cells composing the tumour mass is endowed with the ability to initiate and regenerate the tumour, suggesting the possibility to isolate tumour cells into fractions that are tumour initiating and non-tumour initiating, with only the former capable of sustaining tumour development. The tumour initiating cells have the capacity to self-renew, maintaining and also expanding the tumorigenic subpopulation of cells, but they are also able to differentiate into heterogeneous, non tumorigenic cancer cells that constitute the tumour bulk (Figure 1.2).

Figure 1.2 Models of cancer heterogeneity. According to the stochastic model, tumour cells are biologically equivalent, and their different behaviour is subjective to intrinsic and extrinsic factors. Therefore, tumour-initiating activity cannot be enriched by sorting cells based on intrinsic characteristics. The hierarchy model assumes the existence of biologically distinct classes of cells with differing functional abilities and behaviour. Some cancer cells possess self-renewal ability and are uniquely responsible of tumour initiation and progression, also giving rise to non-tumorigenic progeny that make up the bulk of the tumour. In this perspective, tumour-initiating cells can be identified and purified from the bulk nontumorigenic population based on intrinsic characteristics (adapted from Bonnet and Dick, 1997).



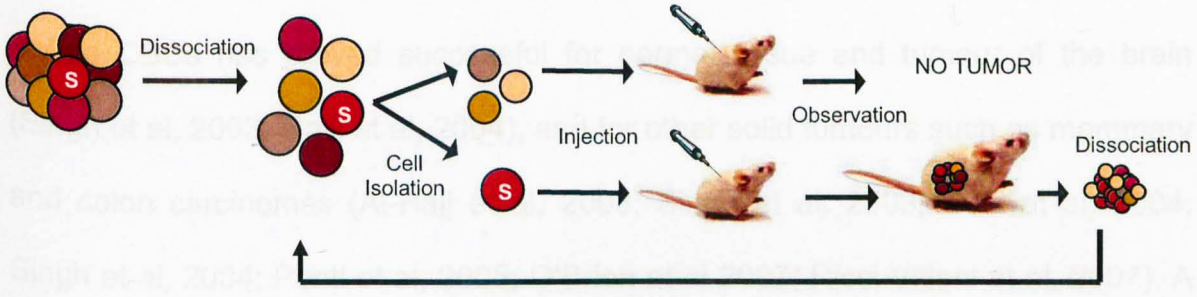
A hierarchical relationship in normal and neoplastic tissues was first demonstrated by experiments taking advantage of radiolabeled leukemic cells.

This technique enabled the precise measurement of proliferation and lifespan of cancer cells. The results clearly showed that human leukemic cells comprised two distinct cellular fractions each displaying different proliferation capacity: a fast cycling subset and a smaller, slow cycling fraction with a dormancy estimated to last from weeks to months. Moreover, the slow cycling fraction was able to generate the fast cycling cells thus suggesting that the slow cycling cells had similar properties to those observed for normal haematopoietic stem cells (HSCs).

Indeed, they were responsible for tumour maintenance and the concept of "cancer stem cells" (CSCs) was thus introduced for the first time. The "CSCs model" implies a hierarchical organization of tumour heterogeneity and in this perspective the tumour is viewed as a caricature of normal tissue development that retains a hierarchical even if aberrant organization with CSCs at the apex (Wang and Dick, 2005).

The currently accepted definition of CSC is a functional one. In fact, CSCs are identified as self-renewing cells that display *in vivo* tumorigenicity and that are able to generate phenotypic diverse progeny. When injected at low dose in immunocompromised animal CSCs are able to re-form tumours with the same features and heterogeneity of the tumour from which they were derived (Clarke, 2005). Tumours generated by stem cells in primary host should be transplantable and maintained over serial *in vivo* passages. This concept led to the alternative definition of putative CSCs as Tumour Initiating Cell (TICs) (Figure 1.3)

Figure 1.3. The gold standard assay for the study of CSCs. The gold standard assay that fulfils the functionally definition of CSCs (S) is the serial transplantation of cancer cell subpopulation in immunocompromised mice. In these assays, cells from mice bearing primary tumours are reisolated and re-injected into secondary recipients and so on. CSCs multipotency is confirmed by their capacity to generate a tumour that mirrors the cellular heterogeneity of the patient original tumour. (Adapted from Schatton and Frank, 2010).



Importantly, the CSC model makes no inferences on the cellular origins of cancers, and it does not require that tumorigenic cells are similar phenotypically, genetically, epigenetically or functionally to the normal stem cells of the same organ. How CSCs are generated is still an open issue. It has been proposed that CSCs may arise by genetic alteration of long-lived normal SCs (Reya et al, 2001) as recently suggested for certain type of lung cancer (Kim et al, 2005).

Nevertheless, recent studies on haematopoietic malignancies indicated that CSCs may arise through oncogenic transformation of progenitors or of even more differentiated cell types (Jamieson et al, 2004; Krivtsov et al, 2006). In addition, CSCs could also originate because of epigenetic transformation occurring in a normal stem cell by a dysfunctional environmental niche (Houghton et al, 2004).

1.1.2.2 Human Cancer Stem Cells: definition of their phenotype

Different approaches, previously used in haematological malignancies, have been employed to select putative CSCs from solid human cancers. CSCs have been selected exploiting their ability to form non-adherent colonies when seeded at clonogenic number in a suitable medium lacking serum but enriched in growth factors (EGF and bFGF). Sphere forming ability as a method to measure and isolate CSCs has proved successful for normal tissue and tumour of the brain (Singh et al, 2003; Galli et al, 2004), and for other solid tumours such as mammary and colon carcinomas (Al-Hajj et al, 2003; Singh et al, 2003; Galli et al, 2004; Singh et al, 2004; Ponti et al, 2005; O'Brien et al 2007; Ricci-Vitiani et al, 2007). A second approach to selectively isolate CSCs is based on their prospective isolation from the tumour bulk using pre-defined markers. Putative CSCs are directly sorted

out from the whole tumour mass using a combination of cell surface markers and the selected populations are then assayed *in vivo* for their ability to form tumours. This method has been successfully applied to tumours of haematological origin and more recently it has also been applied for solid tumours (Bonnet and Dick, 1997; Matsui, et al, 2004; Al-Hajj et al, 2003; Singh et al, 2004; Ma et al, 2007; Li et al, 2007; Prince et al, 2007; Dalerba et al, 2007; O'Brien et al 2007). Concerning solid tumours, the prospective identification of TICs is a challenging task because in most of the cases, little information is available on the hierarchical organization of the corresponding normal tissue and therefore the selection of markers potentially associated with the stem cell compartment becomes a difficult guess.

For some solid tumours such as mammary and colon carcinoma, (Al Haij et al, 2003; Ponti et al., 2005; Ricci-Vitiani et al., 2007) the application of the two methods for CSC isolation, direct isolation using pre-defined markers and *in vitro* expansion of sphere-forming subpopulation, leads to the identification of the same subset of cells further validating the notion that TICs can be pulled out from solid tumour by selecting *in vitro* a population of cells with sphere-forming capacity. Starting by single-cell suspensions obtained from human breast cancer specimens and using flow cytometry–assisted cell sorting, Al-Hajj and colleagues succeed in the isolation of a $CD44^{+}CD24^{-}$ subset of breast cancer cells that exclusively possessed the capacity to generate tumours, when injected at low doses in immunocompromised mice. The xenografted tumours, mirrored the phenotype of the patient's tumour from which they were derived (Al-Hajj et al., 2003). This same cell subset $CD44^{+}CD24^{-}$ was also defined as TICs using the sphere forming assay (Ponti et al., 2005). CD44, either alone or in combination with other markers, has also been proposed to identify the CSC subset in a variety of tumours including

colon ($EpCAM^+CD44^+CD166^+$) (Dalerba et al., 2007), pancreas ($ESA^+CD44^+CD24^+$) (Li et al., 2007), prostate ($CD44^+\alpha2\beta1integrin^{high}CD133^+$) (Collins et al., 2005; Patrawala et al., 2005), head and neck ($CD44^+$) (Prince et al., 2007) and ovarian cancer (Zhang et al., 2008a).

CSCs in human brain tumours expressed the cell surface antigen CD133 (human prominin-1) (Singh et al., 2003). CD133, identified as a cell surface antigen present in HSCs (Yin et al., 1997), is a human five-transmembrane domain glycoprotein whose function is not well understood. With an experimental approach similar to that used in identifying breast CSCs, the injection of $CD133^+$ but not of $CD133^-$ brain tumour cells resulted in xenograft formation. As few as 100 $CD133^+$ cells produced serially transplantable xenografts that were phenotypically and histologically similar to the patients' tumour from which the CD133 cells were derived. Besides brain cancer, CD133 has been also investigated as marker for the CSC population in a variety of human cancers, including colon (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) and lung (Eramo et al., 2008) cancers, hepatocellular carcinoma (Song et al., 2009) and Ewing's sarcoma (Suva et al., 2009). CD133 expression has been also explored in melanoma, with evidence for an enhanced expression of CD133 (associated also to CD166 and of NGFR) in metastatic lesions (Klein et al., 2007). Monzani and colleagues (Monzani et al., 2007) although examining a very limited number of cases, showed that $CD133^+$ cells, isolated from melanoma samples, exclusively displayed the ability to grow as melanospheres and possessed enhanced *in vivo* tumorigenicity as compared to the CD133 negative counterpart.

In pancreatic cancer, CD133 not only identifies CSCs but when coexpressed with CXCR4 it defines CSCs capable of initiating metastatic disease. Thus, while,

both the $CD133^+$ $CXCR4^-$ and the $CD133^+$ $CXCR4^+$ fractions initiate disease at the primary site, only $CD133^+$ $CXCR4^+$ cells were capable to produce metastasis (Hermann et al., 2007). Although CD133 enriches for CSC, it is not yet proved that every $CD133^+$ cell represents a CSC, and the identification of additional CSC specific markers is still needed.

It is probably reasonable to speculate that CSCs should be defined by a combination of several markers. The whole amount of data currently available for colon CSCs, goes in this direction and a recent paper seem to indicate that a combination of CD133, CD44 and CD26 will potentially identify colon CSCs also displaying metastatic capacity (Pang et al, 2010).

Another approach to identify tumorigenic fractions is based on the use of the side population (SP) assay. This assay exploits the inherent feature of stem cells to extrude cytotoxic dyes such as Hoechst33342. This phenotype is due to the efflux pump of the (ATP)-binding cassette (ABC) transporter protein family present on the plasma membrane in diverse cell types. CSCs, extruding Hoechst more rapidly, appear less fluorescent in flow cytometry analysis than the non stem cell counterpart. Hoechst-excluding SP cells sorted from many human established cancer cell lines (Patrawala et al., 2005) were shown to be endowed with enhanced tumour formation capacity *in vivo*. Tumorigenic SP^+ cells were also sorted directly from human tumours. In primary musculoskeletal tumours, ranging from benign to high-grade sarcomas, SP^+ but not SP^- cells showed tumour-initiating capacity; moreover, the percentage of SP^+ cells detectable in the primary tumour was directly correlated with its aggressiveness (Wu et al., 2007). SP^+ cells with tumour initiation capacity and self-renewal ability have been also identified in primary human ovarian cancers (Moserle et al., 2008).

Studies by Schatton and colleagues further support the tumorigenic role of SP in melanoma (Schatton et al., 2008). Instead of studying the SP^+ cells, they looked at the expression of ABCB5, one of the chemoresistance-mediating ABC proteins on the surface of melanoma cells. They showed that ABCB5+ melanoma cells were enriched in tumour-initiating cells as compared with their ABCB5- counterpart. Moreover, the systemic administration of specific anti-ABCB5 antibodies, led to *in vivo* reduction of tumour mass (Schatton et al., 2008).

ALDH1, a member of the ubiquitous aldehyde dehydrogenase (ALDH) family is indeed a functional marker useful for the enrichment of breast cancer stem cells. ALDH1 mediates the conversion of retinol to retinoic acid, a crucial pathway for proliferation, differentiation and survival (Ginestier et al., 2007). In colon cancer, the activity of ALDH1 was responsible for the resistance to cyclophosphamide and the cells expressing ADLH1 were also positive for the colon cancer stem cells markers ESA and CD44 thus confirming that ALDH1 is a functional marker for cancer stem cells (Dylla et al., 2008). Recently, ALDH1 has been reported to select thyroid carcinoma cells with CSC properties and endowed with high metastatic activity (Todaro et al., 2010). As for the $CD133^+$ cells, the $ALDH1^+$ cells are heterogeneous and the expression of other markers identifies a subpopulation of $ALDH1^+$ cells with enhanced tumorigenic capacity. This is the case for CXCR1, the receptor for the IL-8 cytokine, whose expression further defines the breast carcinoma CSCs inside the $ALDH1^+$ subpopulation (Ginestier et al., 2010). Conversely, conflicting results have been reported on the possible role of ALDH1 in defining tumour initiating cells for human melanoma. Recently published results indicate this marker as useful to select melanoma cells with enhanced tumorigenic activity (Boonyaratanakornkit et al., 2010). These data have been only partially

confirmed by Prasmickaite and colleagues. In fact, while they showed that ADLH1$^+$ melanoma cells efficiently generate xenografts which contain both ADLH1$^+$ and ALDH1 negative cells, nevertheless in their system, the ALDH1$^+$ and ALDH1$^-$ melanoma cells displayed similar tumour initiation capacity *in vivo* (Prasmickaite et al, 2010).

Melanoma is a very aggressive tumour, often clinically detectable only in their metastatic forms. Moreover, the rules governing the differentiation pathways of melanocytes from their precursor, namely melanoblasts, are not yet defined. While some results have cast doubts on the hierarchical organization of human melanomas and thus on the existence of a subset of cells selectively endowed with tumorigenic potential (Quintana et al., 2010), other investigators, using prospective isolation of CSCs and FACS sorting or exploiting the sphere formation capacity of CSCs, have provided evidence for the presence of classically defined CSCs in human melanoma (Monzani et al., 2007; Schatton et al., 2008; Roesch et al., 2010; Boiko et al., 2010) and as also shown by results presented in this thesis. However, a consensus on the cell surface markers defining cells with tumorigenic capacity for melanoma has still not been reached. CD20 was firstly proposed to be expressed preferentially on melanoma cells with increased tumourigenicity (Fang et al., 2005), and more recently it has been reported that melanoma CSCs can be isolated as a highly enriched CD271$^+$ population (Boiko et al., 2010).

By analogy with SCs of normal tissues, CSCs are thought to remain quiescent. Thus it has been proposed that CSCs could be isolated from solid tumours using labels that, because of their irreversible binding to the lipid bilayer on cell membrane get equally apportioned between the daughter cells resulting from cell division. Slow cycling CSCs will retain the label and will appear as a subpopulation

with higher fluorescence intensity than the population of dividing cells that lose the dye faster.

Following this approach, PKH67/PKH26 dyes were first used to isolate CSCs from human ovarian cancer and pancreatic adenocarcinoma (Kusumbe and Bapat, 2009). For this last tumour, the slow-cycling CSCs were found to be enriched in $CD44^{+}CD24^{+}$ $CD133^{+}$ $ALDH^{+}$ as compared to the total population or to the rapid dividing cells (Dembinski and Krauss, 2009). Most recently it has also been shown that the PKH26high fraction of human breast cancer is enriched in tumorigenic cells expressing α6 integrin and high level of Delta/Notch-like EGF-Related receptors (DNER) and Delta-Like Ligand (DELL) ($CD49f^{+}$DLLhighDNERhigh) (Pece et al., 2010).

The same experimental approach was applied to human melanoma and led to the identification of a slow-cycling subpopulation of cells required for continuous tumour growth. These slow cycling cells responsible for tumour maintenance *in vitro*, express the H3K4 demethylase JARID1B (Roesch et al., 2010). Although the JARID1B+ cells self-renew and give rise to rapidly proliferating progeny, the capacity of melanoma cells to initiate new tumours in immunocompromised mice is independent of JARID1B expression. Interestingly, JARID1B expression is dynamically regulated and it does not follow a rigid hierarchical model.

Markers associated with tumour initiating cells in different malignancies are summarized in table 1.2.

Table 1.2. Markers used to isolate CSCs from human solid tumours.

Tumour type	Marker	Minimal number of cell for tumour formation	Matrigel	Transplantation site	Mouse strain	Ref.
Breast	$CD44^+CD24^{-/low}$	200	+	Mammary fat pad	NOD-SCID	5-167
	$ALDH^+$	500	+[1]	Mammary fat pad	NOD-SCID	66
	$ALDH^+CXCR1^+$	100	+	Mammary fat pad	NOD-SCID	67
	Label retaining ($PKH26^{high}$) ($CD49f^+DLL^{high}DNER^{high}$)	500	-	Mammary fat pad	NOD-SCID	159
Brain	$CD133^+$ (GBM)	100	-	Brain	NOD-SCID	199
Colon	$CD133^+$	$3x10^3$	-	Subcutaneous	SCID	180
	$EpCAM^{hi}/CD44^+/CD166^+$	200	+	Subcutaneous	NOD-SCID	36
	CD26	$1x10^3$	+	Subcutaneous/ cecal wall	SCID	155
Head&Neck	$CD44^+$	$5x10^3$	+	Subcutaneous	NOD-SCID	170
Ewing's sarcoma	$CD133^+$	$2.5x10^3$	+	Kidney	NOD-SCID	211
Pancreas	$CD44^+CD24^+ESA^+$	100	+	Subcutaneous	NOD-SCID	125
	$CD133^+$	500	-	Subcutaneous	NMR-nu/nu	81
	Label retaining (Dil[4])	$5x10^3$	+	Subcutaneous	SCID	39
Lung	$CD133^+$	10^4	-	Subcutaneous	SCID	50
Liver	$CD90^+$	$5x10^3$	-	Liver	SCID/Beige	130
Thyroid carcinoma	$ALDH^+$	$5x10^3$	-	Subcutaneous	NOD-SCID	220
Sarcomas	Side Population	100	-	Subcutaneous	NOD-SCID	239
Prostate	$CD44^+/\alpha2\beta1integrin^{high}/CD133^+$	100	+	Subcutaneous	NOD-SCID	158
	Side Population	100	+	Subcutaneous	NOD-SCID	157
Ovarian	$CD44^+$ $CD117^+$	100	+	Subcutaneous/i.p[2]	Nude	253
	Side Population	500	+[3]	Kidney capsule	Nude	143
	$PKH26^{high}$	$5x10^3$	-	Subcutaneous/ i.p.	NOD-SCID	113
Melanoma	$CD20^+$	$2x10^5$	+	Subcutaneous	NOD-SCID	53
	CD133	$3.5x10^5$		Subcutaneous	NOD-SCID	140
	$ABCB5^+$	10^6	-	Subcutaneous	NOD-SCID	191
	$CD271^+$	$2x10^4$	-	Subcutaneous	$Rag2^{-/-}\gamma c^{-/-}$ NOD-SCID/IL-$2r\gamma^{null}$	22
	Label retaining ($PKH26^{high}$) $JARIDB1^+$	1	- +[5]	Subcutaneous	NOD/LtSscid/IL-$2R\gamma^{null}$	183

[1] Injected with fibroblasts; [2] Intra peritoneum; [3] Rat tail collage gel; [3] Vybrant_ Dil cell-labeling solution [5] serial transplantation. GBM= glioblastoma multiform; MB=medulloblastoma.

1.1.2.3 Challenging issues of cancer stem cell model

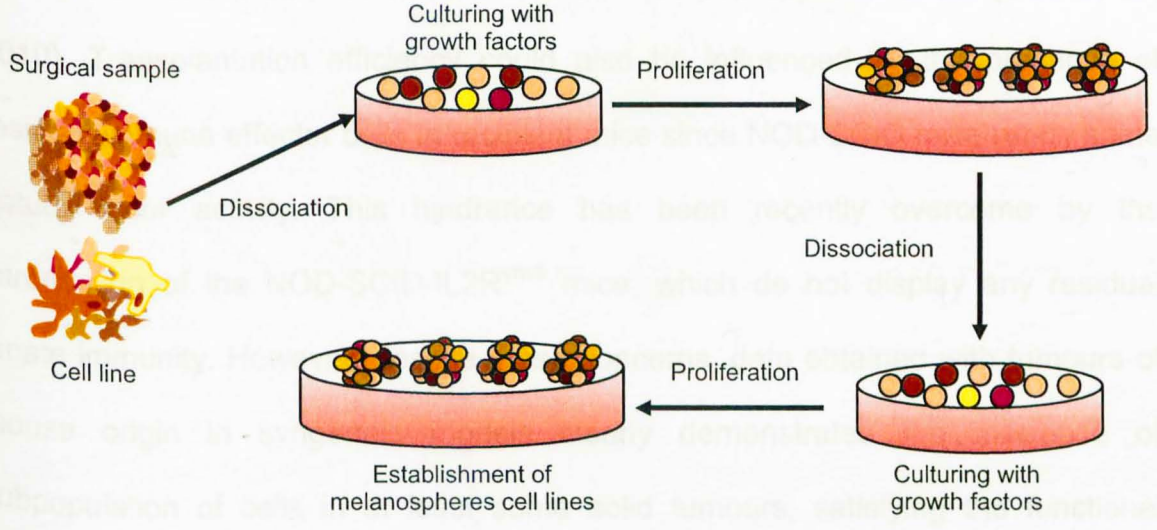
As outlined in 1.1.2.2, CSCs have been indentified and isolated from human tumours mainly by taking advantage of two alternative experimental procedures. One of these approaches directly sorts the putative CSCs from the whole tumour mass using a combination of cell surface markers. The selected populations are then assayed *in vivo* for their ability to form tumours. The application of flow cytometry to stem cell purification is still challenging because of the presence of crucial technical issues that need to be carefully evaluated.

Although flow cytometry offers a sensitive method for cell isolation and purification, occasionally cell aggregates or doublets could interfere with the process of sorting and the sorting conditions chosen for a given cellular subtype could be not well tolerated by other cells of the same organs. In addition, the definition of the cells as high, middle or low regarding the expression of a marker is subjective and can vary depending on the method used for cell and antibody preparation or it could be influenced by the gate setting.

An alternative way to select CSCs is based on their ability to form non-adherent colonies when seeded at clonogenic density in a suitable medium and non-adherent sphere assays are increasingly being used to evaluate stem cell activity in normal tissues as well as in tumours. "Neurospheres" have been widely used to investigate neurogenesis and this approach, extended to the tumours of the entire nervous system, was considered a valuable model subsequently applied to many other solid tumours. Briefly, cells obtained from human cell lines or from surgical specimens by mechanical and enzymatic digestion are plated at low density in specific culture media characterized by the absence of serum and the presence of

growth factors, namely Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF). Clonal density during *in vitro* culture as well as the appropriate concentration of growth factors is crucial for the maintenance of cells with feature of self-renewal. Due to the repeated *in vitro* replications, cells may clearly acquire additional genetic aberrations and this is a drawback of the sphere forming approach in the identification of TICs. However it has been recently reported that neural stem cells propagated *in vitro* for long periods of time maintained a strikingly stable profile with regard to self-renewal, differentiation, growth factor dependence, karyotype, and molecular profiling (Foroni et al., 2007) (Figure 1.4).

Figure 1.4. Sphere assay for isolation of CSCs. Cells obtained from cell lines or from surgical specimens are plated at low plating density in a serum free culture medium supplemented with stem-cell mitogens. Most of the cells die, except those that respond to stem-cell mitogens. These cells proliferate to form floating aggregates named spheres. These spheres can be dissociated into single cells and then re-seeded in fresh medium giving rise to secondary spheres. This procedure can be repeated, resulting in an expansion in the number of cells originated at each passage. (Adapted from Vescovi et al., 2006).



In xenotransplantation assay, cells are orthotopically engrafted into immunocompromised mice that are then assayed at various time points for tumour formation. Tumours engrafted in the primary recipients should be capable of self-

renewal when grafted in subsequent animals. Several critical steps are inside this crucial and fundamental step of TICs identification. First xenotransplantation of viable single cells from solid tumours require modifications of the approaches used for haematological malignancies because of the need to dissociate solid tissues into single cells, thus requiring several manipulations that may affect cell viability or behaviour. Thus, the frequency of CSCs calculated in limiting dilution experiments can be markedly influenced by these parameters.

In addition, the grafting site and the modalities of tumour cell injections, namely injection of tumour cell alone or mixed with Matrigel or with stromal cells of human or murine origin, may indeed affect the efficiency in tumour take. We should then consider wether some murine growth factors, crucial stimuli for stem and progenitor cells in multiple organs, can interact with their equivalent human receptors. Moreover, it is well known that co-injection of tumour cells together with normal fibroblasts increases latency and decreases tumour take, whereas the addition of carcinoma-associated fibroblasts has the opposite effect (Liao et al., 2010). Transplantation efficiency could also be influenced by the presence of residual immune effector cells in recipient mice since NOD-SCID mice retain some natural killer activity. This hindrance has been recently overcome by the introduction of the NOD-SCID-IL2R$^{\gamma null}$ mice, which do not display any residual innate immunity. However, despite these concerns, data obtained with tumours of mouse origin in syngeneic models clearly demonstrated the existence of subpopulation of cells in at least some solid tumours, satisfying the functional criteria of CSCs.

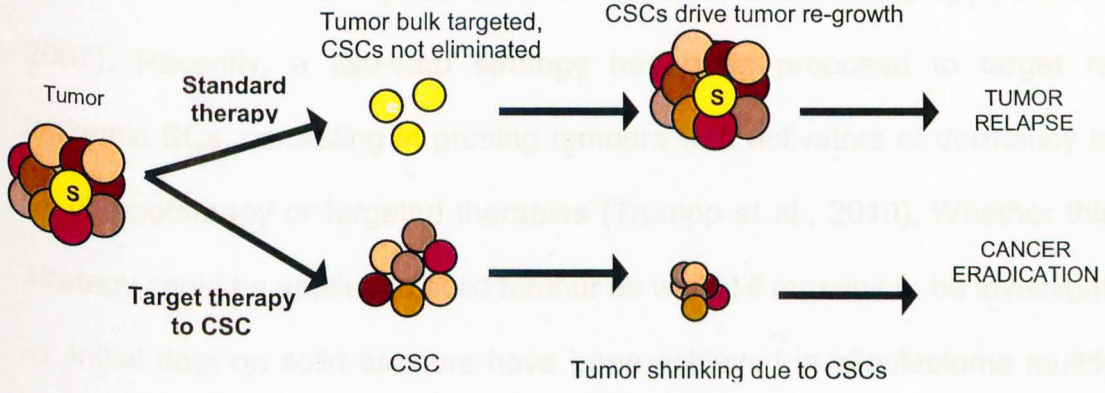
An integrated, multifaceted approach with the development of suitable ex vivo and *in vivo* models will allow better characterization of the CSCs. High-resolution

imaging technologies together with stromal markers will improve our understanding of the cellular niche for different CSCs and will facilitate refinement of transplantation models to more accurately recapitulate the niche for tumorigenic cells.

1.1.2.4 Translational relevance of Cancer Stem Cell Concept

The CSCs theory posits that CSCs are self renewing cancer cells that drive tumour initiation and growth. Thus, it this subset of cells that need to be targeted and eliminated in order to achieve cure, and if a therapy fails to eliminate all self-renewing CSCs, residual surviving CSCs will lead to tumour relapse (Figure 1.5).

Figure 1.5 CSCs and drug resistance. Conventional anti-cancer therapies (e.g. chemotherapy and radiation) can often shrink tumours by targeting tumour bulk, but these therapies fail to target and kill CSCs (S). CSCs subsequently re-drive tumour growth leading to treatment failure and relapse (Adapted from Stemline Therapeutics, Inc. Copyright 2008).



Unfortunately, normal stem cells have evolved multiple mechanisms to protect themselves from toxins and genotoxic stress, which make them relatively resistant to cell killing with cytotoxic agents. The analogies between normal SC and CSCs led to the hypothesis that CSCs may be relatively resistant to common therapeutic agents compared to their more differentiated non-tumorigenic counterparts. This

hypothesis has been confirmed and CSCs present in some established cell lines and long-term xenografts appear relatively resistant to cytotoxic drugs (Ho et al, 2007; Todaro et al, 2007). Importantly this concept has been also verified *in vivo* in humans and frequency of CSCs in tumours of women receiving chemotherapy in the neo adjuvant setting for local advanced breast cancer is enhanced after treatment with cytotoxic agents (Li et al, 2008).

From a clinical perspective, it will be important to elucidate the mechanisms of chemoresistance and radioresistance operating in CSCs. There is also an additional need for tracking residual CSCs following treatment in order to monitor the efficacy of potential anti-CSCs therapies.

CSC resistance to chemo- or radiotherapy can be ascribed to the intrinsic cell cycle kinetics of CSCs. Rapidly dividing cells are more sensitive to cytotoxic therapies than quiescent cells and quiescence is a typical feature of normal stem cells. Mirroring the physiological conditions, leukemic stem cells have been demonstrated to remain quiescent and insensitive to chemotherapy (Ishikawa et al, 2007). Recently, a two-step strategy has been proposed to target resistant leukemic SCs, consisting in priming tumours with activators of dormancy followed by chemotherapy or targeted therapies (Trumpp et al., 2010). Whether this same strategy could be applied to solid tumour as well still remains to be investigated.

Initial data on solid tumours have been achieved in glioblastoma multiform. In fact, it has been demonstrated that glioblastoma CSCs could be induced to differentiate if exposed to bone morphogenic protein 4 (BMP4) thus attenuating their tumour forming capacity (Piccirillo et al., 2006). These results show that the CSC fraction may still possess the ability to respond to biological cues for

maturation that may in the future lead to new non-toxic therapy driving differentiation of malignant tissues.

An additional common mechanism of CSC drug resistance includes the expression of multiple drug resistance transporters (Dean et al., 2005), such as ATP-binding cassette half-transporter proteins (ABCG2 and ABCG5) and multidrug resistance protein 1 (MDR1-ABCB1). Much effort has been devoted to the development of inhibitors of ABC transporters such as ABCB1 and ABCG2 that are highly expressed on CSCs. Initial studies used ABC inhibitors in combination with standard therapies, but they did not produce convincing results (Dean et al., 2005), probably due to the low activity of the inhibitors and their difficult in interacting with the chemotherapeutics or because of the presence of additional drug transporters not targeted by the inhibitors. Alternatively, CSC resistance to cytotoxic agents includes other crucially active pathways such as enhanced DNA repair capacity and the expression of specific drug detoxifying enzymes such as ALDH1 (Ginestir et al, 2007).

Reactive Oxygen Species (ROS) are critical mediators of radiation-induced cell killing but stem and early progenitor cells of different tissues contain lower levels of ROS than they their differentiated progeny, suggesting a possible cause of radioresistance. Human mammary CSCs contain lower ROS levels than more differentiated tumour cells, and accumulate less DNA damage upon irradiation (Diehn et al., 2009).

It is also likely that CSCs employ a combination of mechanisms that make them relatively resistant to therapies compared to their progeny, which could even vary between tumour types. The identification of the specific resistance mechanisms at

work on these cells will allow rational design of chemo- and radio-sensitization protocol that could lead to higher tumour control and cure rates.

1.1.2.4.1 Targeting CSCs

Surface markers used to isolate CSCs may represent suitable targets to develop monoclonal antibody based therapies specific for CSCs but sparing non-tumorigenic cells. CD44 is a CSC marker shared by many different human tumours and although its role in CSC biology remains unclear, CD44 enriches for tumorigenic cells in breast (Al-Hajj et al., 2003), prostate (Collins et al., 2005), colon (Dalerba et al., 2007), head and neck (Prince et al., 2007), pancreas (Hermann et al., 2007) and liver tumours (Yang et al., 2008). Treatment of animals with anti-CD44 prevents serial tumour formation in mouse model of human AML (Jin et al., 2006), but given the relatively diverse tissue and cell types expressing CD44, it remains unclear if this antigen will be useful in clinical settings. Similarly, targeting $ABCB5^{+}$ melanoma cells with systemic administration of a monoclonal antibody inducing antibody-dependent cell mediated cytotoxicity led to a reduction of tumour mass in mice xenografted with human melanoma ABCB5+ (Schatton et al., 2008).

Although associated with the CSCs phenotype in many human cancers, CD133 is not a reliable marker due to the technical limitation of its detection. CD133 expressing cells have been isolated using the monoclonal antibodies CD133/1 (AC133) and CD133/2 (AC141) recognizing different glycosylated epitopes that are still poorly characterized. Furthermore, CD133 expression might be regulated by environmental factors such as oxygen concentration (Bidlingmaier et al., 2008).

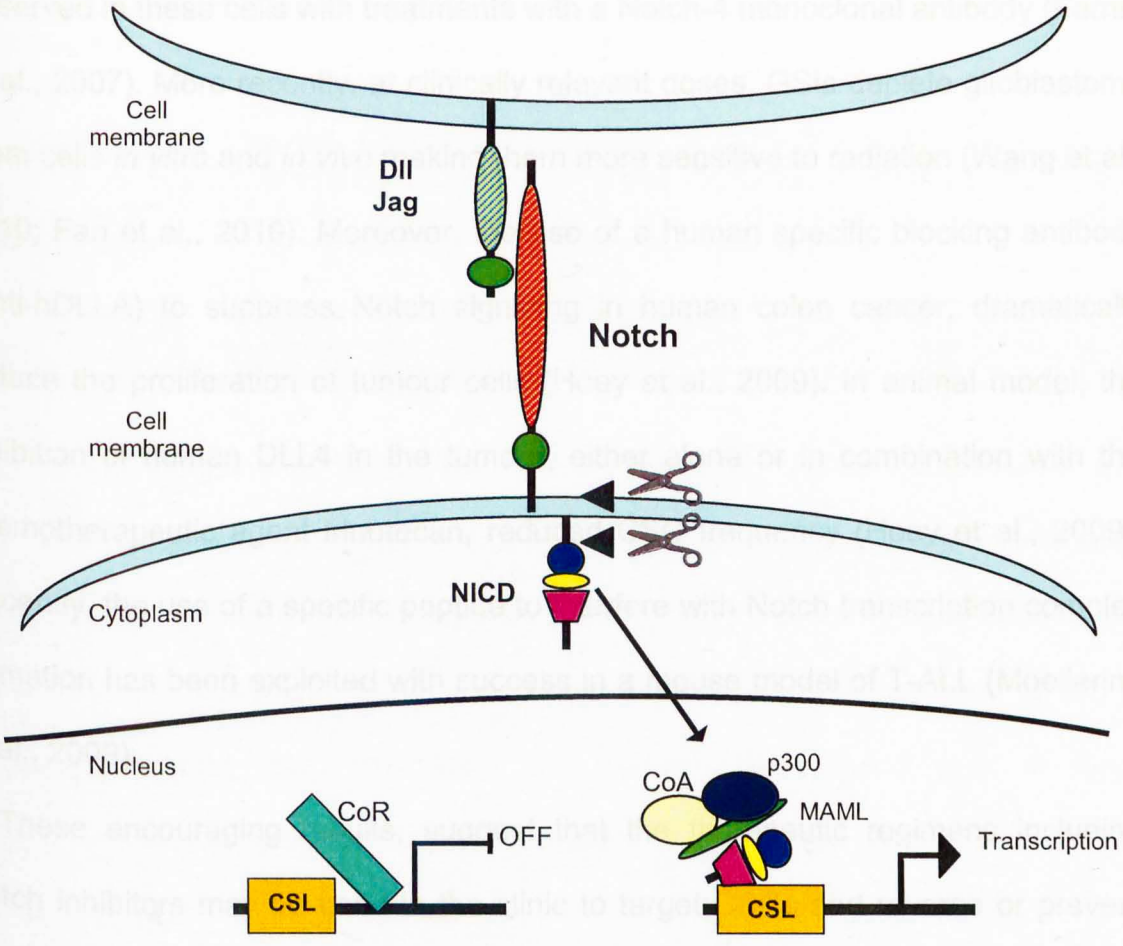
The concept that cancer is a "developmental disease", in which normal developmental pathways have been altered by oncogenic processes, was firstly proposed several decades ago (Pierce and Speers, 1961).

In recent years, it has been shown that embryonic signaling pathways such as Notch, Wnt/β-catenin, and Hedgehog (Hh), regulate adult stem cell self-renewal and survival in the haematopoietic system, skin, nervous system and breast (Kopper et al., 2004). More interestingly, these very same pathways are often abnormally activated in cancer.

The Notch pathway is a short-range communication system in which contact between a cell expressing a membrane-associated ligand and a cell expressing a transmembrane receptor sends to the receptor-expressing cell (and possibly both cells) a cell fate regulatory signal. This signal takes the form of a cascade of transcriptional regulatory events that affect the expression of hundreds of genes, and has profound, context-dependent phenotypic consequences. Mature Notch-receptors (in mammals Notch-1 through-4) are non covalent eterodimers consisting of an extracellular and a transmembrane subunit.

In mammals Notch receptors (Notch1-4) interact with surface bound or secreted ligands (Delta like 1-3-4; Jagged 1-2). Upon ligand binding, Notch receptors are activated by serial cleavage events involving members of the ADAM protease family followed by intramembranous cleavage regulated by γ–secretase. After cleavage, the intracellular domain of Notch (D) translocates into the nucleus to act on downstream targets (Figure 1.6).

Figure 1.6 Notch signaling pathway. Notch signaling starts upon ligand-receptor interaction which induces successive proteolytic cleavages. Metalloproteases of the ADAM family mediate the first cleavage in the extracellular domain, and the subsequent second cleavage in the transmembrane domain is made by a γ-secretase activity of presenilins (PS). This second cleavage frees the intracellular domain of Notch (NICD) that subsequently moves to the nucleus where it forms a binary complex with CSL, leading to transcriptional activation by dislodgment of co-repressor (CoRs) and synchronized recruitment of different co-activators (CoA), including mastermind like proteins (MAML) and p300. Dll= delta-like ligand; Jag= Jagged. (Adapted from ISREC research groups, Radtke 2010).



Notch signaling has been shown to play a pivotal role in cell fate determination in neural, haematopoietic and embryonic stem cells and it has been shown to be involved in the biology of several solid tumours (Allenspach et al., 2002). In agreement with this central role of Notch signaling in the control of SC homeostasis, the Notch pathway is one of the most intensively studied putative therapeutic targets in CSCs, and several Notch inhibitors are being developed. γ-secretase inhibitors (GSIs) capable of blocking Notch receptor cleavage and

pathway activation have been under clinical evaluation for the treatment of malignancies such as gliomas (Fan et al., 2010) or T-cell acute lymphoblastic leukaemia (T-ALL) (Tammam et al., 2009) (Table 1.3). GSIs have been able to appreciably decrease breast CSC self-renewal *in vitro*, analogously to what is observed in these cells with treatments with a Notch-4 monoclonal antibody (Farnie et al., 2007). More recently, at clinically relevant doses, GSIs deplete glioblastoma stem cells *in vitro* and *in vivo* making them more sensitive to radiation (Wang et al., 2010; Fan et al., 2010). Moreover, the use of a human specific blocking antibody (anti-hDLLA) to suppress Notch signaling in human colon cancer, dramatically reduce the proliferation of tumour cells (Hoey et al., 2009). In animal model, the inhibition of human DLL4 in the tumour, either alone or in combination with the chemotherapeutic agent irinotecan, reduced CSC frequency (Hoey et al., 2009). Recently, the use of a specific peptide to interfere with Notch transcription complex formation has been exploited with success in a mouse model of T-ALL (Moellering et al., 2009).

These encouraging results, suggest that the therapeutic regimens including Notch inhibitors may be used in the clinic to target CSCs and reverse or prevent chemo- or radio-resistance. Some of the now available investigational Notch inhibitors are already in the clinic (Table 1.3); GSIs inhibitors have the advantages of relative ease of administration, oral bioavailability, and low cost. Whether intermittent Notch inhibition will be sufficient for effective CSCs targeting in patients is still an open question. If continuous inhibition is necessary for optimal results, systemically delivered GSIs may be disadvantageous, because of their off-target effects mainly related to intestinal toxicity.

Table 1.3. Notch-targeting agents. (Adapted from Pannuti et al., 2010).

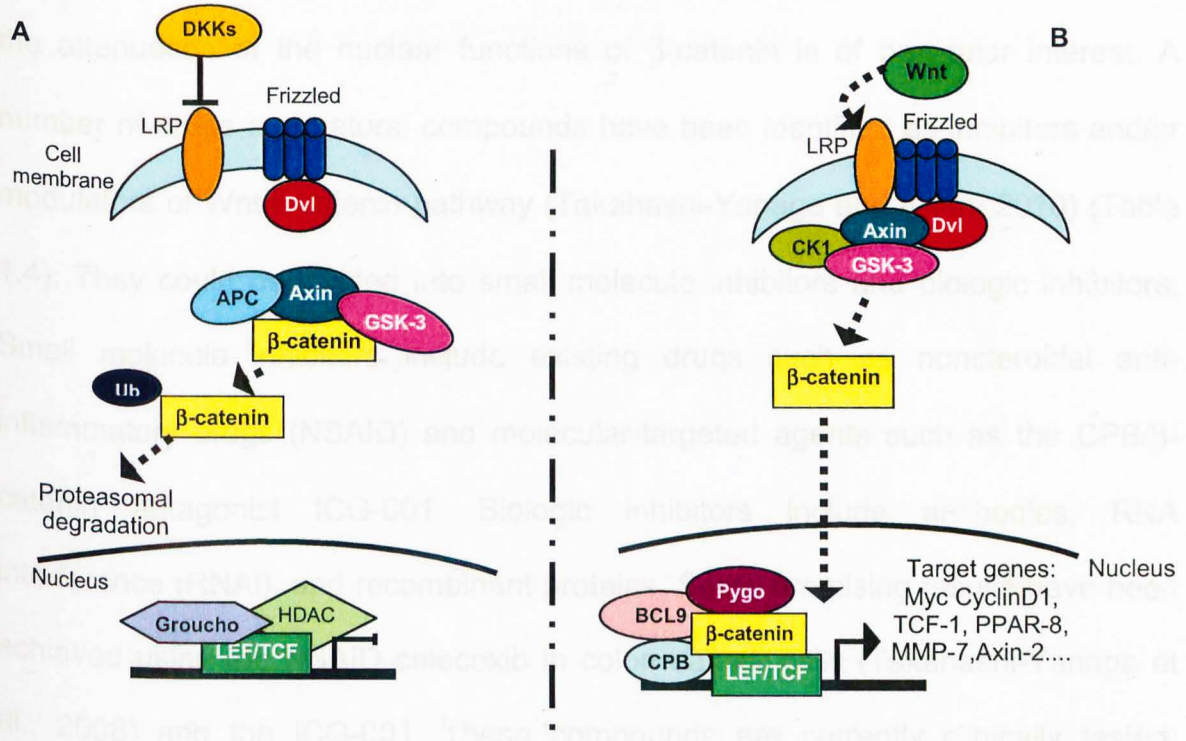
Agent	Mechanism	Targets	Development Phase
GSIs	Inhibition of final Notch cleavage by γ-secretase	All 4 Notch paralogs; Notch ligands; multiple other □-secretase substrates	1-2
MAML1-stapled peptide	Interference with Notch nuclear co-activator MAML-1	All Notch paralogs; potentially other nuclear transcription factors that use MAML1	Preclinical
Notch mAbs	Interference with ligand-induced Notch subunit separation	Specific for individual Notch receptors	Preclinical
DLL4 mAbs	Interference with ligand-receptor interaction	Specific for Delta-4 ligand	Preclinical
Notch soluble receptors decoy	Interference with ligand-receptor interaction	Relatively specific for Notch paralogs potential pan-Notch inhibition	Preclinical
siRNA, miRNA based therapeutics	Interference with expression of Notch signaling components	Specific for target mRNA	Preclinical

The Wnt signaling pathways have been conserved throughout evolution and they regulate cell proliferation, morphology, motility, and fate during embryonic development. These pathways also play important roles throughout adult life to maintain homeostasis of tissues including skin, blood, intestine and brain by regulating somatic stem cells and their niches. Aberrant regulation of these pathways leads to neoplastic transformation in the same tissue (Taipale and Beachy, 2001).

Among the described Wnt signaling pathways the Wnt/β-catenin pathway is the best characterized. The activity of the Wnt/β-catenin pathway is dependent on the amount of β-catenin in the cytoplasm. Normally cytoplasmatic β-catenin is maintained at a low level through ubiquitin-proteasome-mediated degradation, which is regulated by a complex containing axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). Upon binding of Wnt proteins to a receptor complex comprised of Frizzleds/low-density lipoprotein receptor-related protein (Fz/LRP), the cytoplasmic disheveled (Dvl) is phosphorylated thereby

inhibiting GSK-3β, resulting in the accumulation of nonphosphorylated β-catenin in the cytoplasm. Nonphosphorylated β-catenin avoids degradation and translocates into the nucleus. In the nucleus, β-catenin in the classical Wnt signaling cascade forms a complex with members of the T-cell transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors. To generate a transcriptionally active complex, β-catenin recruits the transcriptional co activators, cAMP response element-binding protein (CREB)-binding protein (CBP) or its closely related homolog, p300 as well as other components of the basal transcription machinery, leading to the expression of a host of downstream target genes. Secreted Wnt inhibitor factor 1 (WIF1) and secreted Frizzled-related proteins (SFRP), soluble Wnt receptors and competitors of Fz, respectively, are Wnt target genes that function as endogenous inhibitors of the Wnt signaling pathway (Figure 1.7).

Figure 1.7. The canonical Wnt-β-catenin pathway signaling. A. In the absence of Wnt ligands, β-catenin is recruited into a complex with APC and the axins. Following phosphorylation of β-catenin by the kinases casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β), and subsequent ubiquitylation, β-catenin is proteasomally degraded. Low cytoplasmic levels of β-catenin ensure transcriptional repression of Wnt target genes by recruitment of the corepressor Groucho to LEF (lymphoid enhancer factor)–TCF (T-cell factor) transcription factors. **B.** In the presence of Wnt ligands, LDL-related receptor protein 5 (LPR5) and LRP6 are phosphorylated by CK1γ and GSK3β, and Dishevelled (DVL) molecules are recruited to the plasma membrane to interact with Frizzled (FZD) receptors and other DVL molecules. These Interactions lead to the inactivation of the destruction complex and subsequently to the stabilization of β-catenin and its translocation to the nucleus. In the nucleus, β-catenin forms a transcriptionally active complex with LEF and TCF transcription factors by dislocation of Grouchos and HDAC and interactions with co-activators such as B-cell lymphoma 9 (BCL9), Pygopus (Pygo) and CREB binding protein (CBP). (Adapted from Klaus and Birchmeier, 2009).



The Wnt/β-catenin pathway has been demonstrated to regulate pluripotency in embryonic stem cells (Sato et al., 2004) and it is critical for the fate decision in neural crest stem cells (Hari et al., 2002). This pathway plays an important role in the regulation of the haematopoietic stem cell self-renewal (Staal and Luis, 2010) and it is also involved in the maintenance of mammary (Lamb et al., 2007) and epithelial stem cells (Gu and Watanabe, 2010). Because of the analogies existing between normal and CSCs, it has been suggested that the Wnt/β-catenin pathway could also have a role in the regulation of CSCs. Initial evidence from epithelial

cancers pointed out that an abnormal activation of this pathway is associated with the acquisition of hyperproliferation and self-renewal by intestinal crypt progenitor cells (Radtke and Clevers 2005) and by luminal progenitor cells during oncogenesis (Zhang et al., 2008b); moreover, Wnt signaling has also been recently shown to be essential for the renewal of chronic CML stem cells *in vivo* (Zhao et al., 2007).

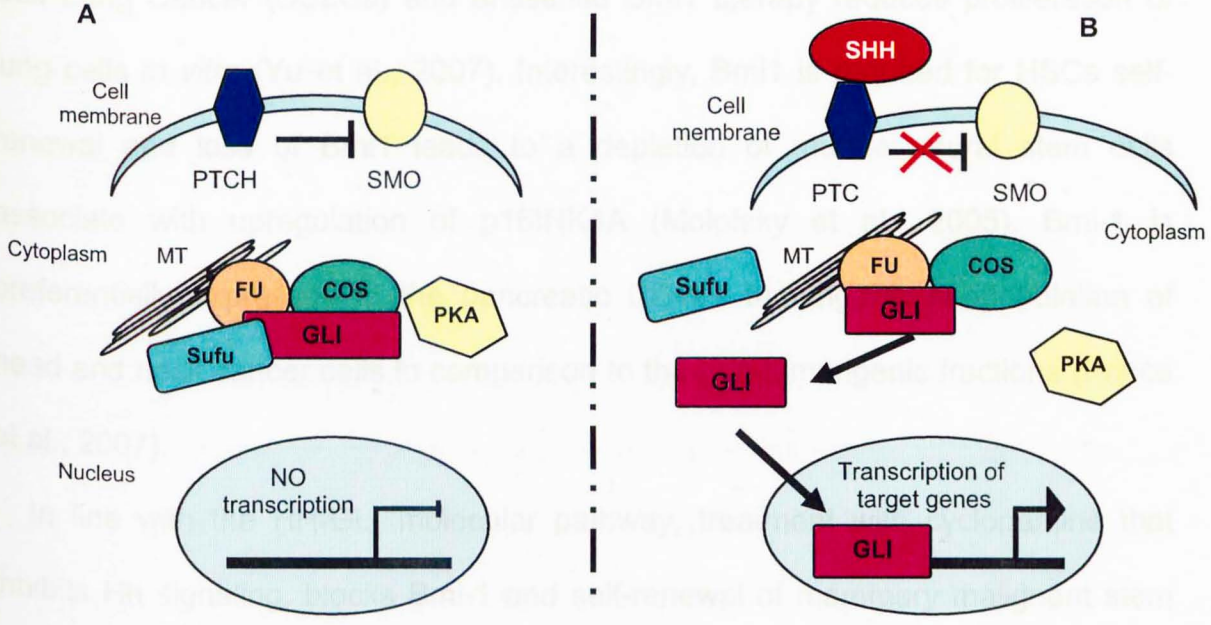
Given the fact that multiple mutations lead to nuclear translocation of β-catenin, the attenuation of the nuclear functions of β-catenin is of particular interest. A number of drugs and natural compounds have been identified as inhibitors and/or modulators of Wnt/β-catenin pathway (Takahashi-Yanaga and Kahn, 2010) (Table 1.4). They could be divided into small molecule inhibitors and biologic inhibitors. Small molecule inhibitors include existing drugs such as nonsteroidal anti-inflammatory drugs (NSAID) and molecular-targeted agents such as the CPB/β-catenin antagonist ICG-001. Biologic inhibitors include antibodies, RNA interference (RNAi), and recombinant proteins. Some promising results have been achieved using the NSAID celecoxib in colon cancer cells (Takahashi-Yanaga et al., 2008) and the ICG-001. These compounds are currently clinically tested. Additional encouraging evidence on the utility of monoclonal antibody directed against Wnt-2 is provided in preclinical models where treatments with this agent have led to tumour control in lung, melanoma, colon and hepatocellular xenografted human tumours (You et al., 2004; Shi et al., 2007; Wei et al., 2009). However no data are available on the effects of this antibody treatment on the CSCs subpopulation.

Table 1.4. Wnt-targeting agents. (Adapted from Takahashi-Yanaga and Kahn, 2010).

Agent	Targets	Development Phase
NSAIDs	β-catenin/TCF	Clinical (Calecoxib)
Vitamins	β-catenin	Clinical
Poliphenoles	Unknown	Preclinical
Molecular targeted drugs	β-catenin/TCF	Discovery-preclinical
	CBP	Phase I
	Dvl	Discovery
	Axin	Discovery
	Porcupine	Discovery
	Tankyrase 1&2	Discovery
Antibodies	Wnt proteins	Preclinical
Recombinant proteins	WIF1 and SFRPs	Preclinical
RNA interfering molecules	Wnt proteins	Preclinical

Many human cancers have been shown to require the activity of the **Hedgehog-GLI** (HH-GLI) signaling pathway for sustaining their growth (Ruiz et al., 2007). Interestingly, HH-GLI not only controls the survival and the proliferation of the bulk of the tumour but it is also required for cancer stem cell survival in leukaemias (Dierks et al., 2009), gliomas (Bar et al., 2007), colon (Varnat et al., 2009), breast (Liu et al., 2006) and pancreatic cancers (Li et al., 2009). HH-GLI signaling is a complex pathway that involves multiple factors even though the core pathway has few components. Briefly, secreted HH glycoproteins bind and inactivate the 12-transmembrane protein PATCHED1 (PTCH1), which itself normally inhibits the activity of the 7-transmembrane G-protein coupled receptor-like protein SMOOTHENED (SMO). Thus, upon presence of the ligand, SMO is free from PTCH1 inhibition and it sends a signal leading to the activation of the GLI transcription factors. Upon HH-SMO signaling, the GLI proteins cease to act as repressors and turn into full-length activators of target genes (Figure 1.8).

Figure 1.8. The Hh-signaling pathway. A. In the absence of Sonic hedgehog (SHH), Patched 1 (Ptc1) inhibits Smoothened (Smo). The Gli proteins are phosphorylated by protein kinase A (PKA), leading to their cleavage by the proteasome and the formation of carboxyl-terminus-truncated repressor Glis, which move to the nucleus and repress the Gli-dependent transcription of target genes. **B.** When Hh binds Ptc, its interactions with Smo are altered such that Smo is no longer inhibited. The macromolecular complex that is associated with the microtubules (MT), and includes Su(fu) (Suppressor of fused), Fu (Fused), PKA, the Gli proteins and other possible components acts to produce labile Gli activators. These are imported into the nucleus and transactivate target genes. This leads to Ci/Gli protein entering the nucleus and acting as a transcriptional activator for the same genes it represses when Ptc is free to interact with and inhibit Smo. The regulation of Gli activity takes place at many levels, including nuclear export and the presence of positive or negative cofactors. In addition, SHH activates the transcription of Ptc1, which, when overexpressed, attenuates the SHH response by preventing SHH from binding Ptc1 molecules within the receptor complex. (Adapted from Ruiz i Altaba et al., 2002).



Targeting Hedgehog-GLI signaling could be a very powerful anti-cancer strategy also because GLI integrates signaling not only dependent from Hedgehog, but it could also be regulated by major human oncogenes (e.g. RAS and AKT) or tumour suppressors (e.g PTEN, p53) (Mas and Ruiz i Altaba, 2010).

Some results have been recently achieved targeting HH-GLI on CSCs. Inhibition of Hh signaling has been shown to affect tumour initiation and systemic metastasis in orthotropic xenografts of human pancreatic cancer with minimal effect on the primary tumour volume, but leading to significant reduction of CSC fraction selected by ALDH expression (Feldmann et al., 2008). CSCs depletion by Hh

signaling inhibition has been also obtained from medulloblastoma (Rudin et al., 2009), glioblastoma (Bar et al., 2008) and breast cancer (Tanaka et al., 2009). An important downstream target of the Hh pathway is the bone morphogenic insertion-1 (Bmi1), a member of the Polycomb group protein family of transcriptional repressors. Bmi1 targets genes such as p16INK4A and p14ARF preventing stem cell senescence. It has been claimed that Bmi1 is expressed in almost all Small Cell Lung Cancer (SCLCs) and antisense Bmi1 therapy reduces proliferation of lung cells *in vitro* (Yu et al., 2007). Interestingly, Bmi1 is required for HSCs self-renewal and loss of Bmi1 leads to a depletion of murine neural stem cells associate with upregulation of p16INK4A (Molofsky et al., 2005). Bmi-1 is preferentially expressed in the pancreatic CD44+ tumorigenic subpopulation of head and neck cancer cells in comparison to the non-tumorigenic fractions (Prince et al., 2007).

In line with the HH-GLI molecular pathway, treatment with cyclopamine that inhibits Hh signaling, blocks Bmi-1 and self-renewal of mammary malignant stem cells (Liu et al., 2006). On the basis of these crucial data, several compounds working as hedgehog pathway antagonists are currently being tested in the clinical setting (Table 1.5).

Table 1.5. Hedgehog-GLI-targeting agents. (Adapted from Mas and Ruiz i Altaba, 2010).

Agent	Targets	Development Phase
GDC0449	SMOH	Phase II
BMS-833923	SMOH	Phase I
LDE225	SMOH	Phase I-II
IPI-926	SMOH	Phase I
Zerumbone	Gli	Discovery
Starusporinone	Gli	Discovery
Acryfalvin C	Gli	Discovery
Physalin B-F	Gli	Discovery
Robotnikinin	Shh	Discovery

Components of PI3K pathway, including PTEN, Akt and mTOR are critical regulators of both normal SC function and tumorigenesis. Intriguingly, inactivation of some pathway components, like PTEN has an opposite effect on normal HSCs and CSCs. PTEN has an essential role in restricting the activation of HSCs, in lineage fate determination and in the prevention of leukemogenesis. In fact PTEN deletion leads to impair reconstitution of bone marrow after HSC transplantation. In contrast, CSCs arise and expand in number after PTEN deletion in murine model of leukaemia. Furthermore, the CSCs were found to be sensitive to the mTOR inhibitor Rapamycin, while the normal HSCs were not (Yilmaz et al., 2006). The PTEN/Akt/mTOR pathways have also been described to confer resistance to conventional therapies and play a central role in the viability and maintenance of prostate epithelium progenitor cells (Zhang et al., 2006b) and of CSCs in breast and prostate cancer (Zhou et al., 2007; Dubrovska et al., 2009). Using a murine lymphoma model it was firstly shown that using the mTOR inhibitor Rapamycin in combination with standard chemotherapeutics (doxorubicin) it was possible to efficiently eradicate tumour cells (Wendel et al., 2004). Moreover, combination therapy inhibiting Hh and mTOR signaling, together with standard chemotherapy

(gemcitabine), was effective in selectively eliminating CSCs in pancreatic cancer (Mueller et al., 2009).

There is increasing interest in the possibility of exploiting the putative CSC niche for drug targeting. The existence and the architecture of the CSC niche remain elusive although there is a substantial evidence for an instructive role of the tumour microenvironment. Nevertheless, aberrant stem cell niches may result in disease, as exemplified by an altered haematopoietic stem cell niche leading to the development of myeloproliferative diseases (Walkely et al., 2007). Moreover, glioblastoma and medulloblastoma CSCs appear to be maintained by signals from an aberrant vascular niche that mimics the normal stem cell niche (Bao et al., 2006; Calabrese et al., 2007). These studies suggest that brain CSCs have potent angiogenic activity. In fact, intraperitoneal administration of anti-angiogenic therapy with Bevacizumab, a vascular endothelial growth factor (VEGF)-neutralizing monoclonal antibody, markedly reduce CSCs tumourigenicity *in vivo* (Folkins et al., 2007). Recent evidence also suggest the possibility of using antibodies directed against molecules that could play a crucial role in mediating CSC drug resistance such as cytokines and chemokines. This in accordance to what has been observed in colon CSCs, that could became drug sensitive if treated with an IL-4 neutralizing antibody (Todaro et al., 2007).

1.2 MELANOMA

1.2.1 Epidemiology

Cutaneous melanoma, also referred to as "malignant melanoma", represents 3-7% of all skin malignancies and is the most serious form of skin cancer because of its propensity for early invasion and widespread metastasis. With early detection and treatment, the cure rate of melanoma is about 95%, but advanced melanoma appears to be resistant to conventional therapies and, despite the overall advances in the fields of tumour biology and oncology therapies, prognosis for patients with widely metastatic disease remains poor, with a median survival rate of 6 months and 5-year survival rate of less then 5% (Cummins et al, 2006).

The incidence of malignant melanoma is rapidly increasing, from 1975 to 2005, data from National Cancer Institute Surveillance Epidemiology and End Results (SEER) show that incidence of melanoma has nearly tripled in the United States.

Based on the SEER data for 2001 to 2005, the median age at diagnosis for melanoma was 59 and the median age at death was 68. Additional breakdown in incidence and mortality by age group is shown in Table 1.6. Melanoma is several times more common in whites compared with more highly pigmented ethic groups and men are 1.5 times more likely to develop melanoma than women (Table 1.7).

Table 1.6: Incidence and death for melanoma by age. Data from National Cancer Institute. Surveillance, Epidemiology and End Results (SEER). (Cho and Chiang, 2010).

Age	Incidence (%)	Deaths (%)
<20	0.9	0.1
20-34	8.1	2.9
35-44	12.9	7.2
45-54	18.9	15.0
55-64	19.5	18.8
65-74	17.8	21.3
74-84	16.4	23.6
>85	5.5	11.0

Table 1.7: **Incidence and death rates for melanoma by age and sex in the period 2001-2005**. Data from National Cancer Institute. Surveillance, Epidemiology and End Results (SEER) (Adapted from Cho and Chiang, 2010).

	Incidence Rate by Race		Deaths rates by Race	
Race or Ethnicity	*Male (per 100.000)*	*Female (per 100.000)*	*Male (per 100.000)*	*Female (per 100.000)*
All races	24.6	15.6	3.9	1.7
White	28.5	18.5	4.4	2.0
Balck	1.1	0.9	0.5	0.4
Asian or Pacific Islander	1.6	1.3	0.5	0.3
American Indian or Alaska Naive	3.9	2.6	1.5	0.7
Hispanic	4.8	4.9	0.9	0.6

1.2.2 Risk Factors

The overall risk of developing melanoma during lifetime increases when multiple risk factors are present. Having one or two risk factors increase the risk of melanoma by two-to four-fold, and more than three risk factors can increase the risk by more than twentyfold (Cho et al 2005).

1.2.2.1 Environmental Factor

Risk factors can be divided into environmental factors and host factors. The main environmental risk is ultraviolet radiation (UVR); as reported in many studies, history of sunburns confers twice the risk of developing melanoma compared with no prior history of sunburns (Cho et al, 2005). Moreover, aged people with a childhood history of sunburns are at even higher risk (Elwood and Jopson 1997). Australia and New Zeeland have the highest incidence and mortality rates for melanoma in the world with the estimated risk to develop melanoma over a lifetime of 3.3 and 5.7% respectively (Jones et al, 1999) because at their latitudes sun is more direct and UVR levels are higher.

1.2.2.2 Host Factors

Specific phenotypic characteristics such as red or blond hair, skin type, sun sensitivity and presence of freckles are associated to an enhanced risk of developing melanoma (Veierod et al, 2003). Skin type is defined by the quantity and the type of melanin. Melanin, a pigment produced by the skin melanocytes naturally protects against UV damage, absorbing UV and neutralizing free radicals produced by the radiation. Two types of melanin exist in the skin: eumelanin and pheomelanin. Eumelanin is responsible for gray, black, yellow colours found in hair and skin and confer greater UVR protection; pheomelanin is responsible for more pink and red colours. The ratio between eumelanin and pheomelanin is controlled by the melanocortin 1 receptor (MC1R) that induces an increase in eumelanin production. People with red hair have diminished MC1R function and in their skin pheomelanin is the predominant form of melanin; impaired function of this receptor is considered an independent risk factor for melanoma developing (Rees 2004).

Family history is also strong risk for melanoma in fact, patients with a first-degree relative with melanoma have a twofold higher risk of developing melanoma compared to patients with a non-family history of melanoma (Cho et al, 2005). Familial melanoma represents about 10% of all melanoma cases (High and Robinson, 2007) and different susceptibility genes have been described including cyclin-dependent kinase 4 (*CDK4*) alternative reading frame (*ARF*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and MC1R (Table 1.8). A genetic predisposition to melanoma may also occur in a patient without a family history that may acquire a new mutation.

Melanocytic nevi are benign accumulation of melanocytes and may be congenital or acquired. The risk of developing melanoma increases with the number of nevi on the body. Having more then ten nevi greater then three millimetres on the arm can increase the risk of melanoma development up to 4.7 fold on the trunk, shoulder, back or abdomen; 3.5 fold on the head and neck, 2.5 on the upper extremity and 2 fold on the lower extremity (Cho et al, 2005). Larger nevi are associated with a greater risk of melanoma (Watt et al, 2004). Dysplastic nevi, that are melanocytic nevi with cytological atypia, are considered an independent risk factor for melanoma and it has been recently reported that individuals with five or more dysplastic nevi had six times the risk of developing melanoma compared to subjects with no atypical nevi (Gandini et al, 2005).

Tab. 1.8 Susceptibility genes in familial melanoma. (Adapted from High and Robinson, 2007).

Gene	Protein	Chromosomal locus	Melanoma risk	Penetrance (high/low)
MC1R	MSH-α receptor	16q24.3	2- fold to 3.6-fold increased risk	Low
CDKN2A	INK4a ($p16^{INK4a}$)	9p21(exons 1α,2,3)	67% lifetime risk; 35-fold to 70-fold relative risk	High
CDKN2A	ARF ($p14^{ARF}$)	9p21(exons 1β,2,3)	Unknown	High
CDK4	CDK4	12q13	Unknown	High

1.2.3 Diagnosis and prognosis of melanoma.

1.2.3.1 Diagnosis criteria and subtypes of melanoma

Pigmented lesion need to be evaluated firstly considering the "ABCDE system", that simply evaluates Asymmetry (A), Border irregularity (B), Colour variation (C), Diameter (D) and Evolving (E) of the lesion, but a biopsy with a histopathologic evaluation is mandatory for the correct diagnosis.

Melanoma could be divided into four main subtypes, distinguished by clinical and pathological growth patterns.

❖ Superficial spreading melanoma

Superficial spreading melanoma (SSM) is the most common type of melanoma in United States, accounting for about 70% of all diagnosed melanoma cases. This type of melanoma occurs slightly more often in females than males, it can arise at any age and it is the leading cause of death in young adults. SSM tends to occur on sun-exposed skin, and it appears most commonly on the legs in females and on the backs of males (Newell, 1988). In the early stages, SSM usually appear as a flat spot similar to a freckle spreading sideways on the skin. Over time, the lesion grows acquiring the typical features of SSM lesions (irregular borders, various shades of black, brown, gray, blue, pink, red or white with possible variation in colour within the lesion). While, growing, the lesion may have areas of inflammation and the region around the lesion may begin to itch.

❖ Nodular melanoma

Nodular Melanoma (NM), is the most aggressive kind of melanoma and accounts for about 15% of all melanoma diagnosed in the United States. It can appear anywhere on the skin and occurs more often in males than females, preferentially developing in people aged 50 and older. NM tends to growth more rapidly in thickness penetrating the skin than in diameter and can appear in regions lacking a previous lesion; NM is most often darkly pigmented and commonly present ulcerationing and bleeding lesions. The most common sites for NM are trunk, head and neck (Lens 2008).

❖ Lentigo Maligna Melanoma

Lentigo maligna melanoma (LMM) typically occurs on sun-damaged skin in the middle aged and elderly, especially on the face and accounts 10% of the melanoma diagnosed in the United States.

LMM begins as a spreading, flat, patch with irregular borders and variable shades of brown, for this reason, can be easily mistaken for an "age spot" or "sun spot" in its early phases. As the lesion evolves, both the pigmentation and the margins become more irregular, with the possible appearance of irregular dark nodules within the borders that represent the invasive tumour. Long-term cumulative rather than intermittent sun exposure is thought to confer the higher risk for developing LMM that appear almost uniquely on the sun-exposed skin of the head and neck (Lens, 2008).

❖ Acral Lentiginous Melanoma

Acral Lentiginous Melanoma (ALM) counts for the 5% of the melanoma diagnoses in the United States but is the most common form of melanoma in Asians and in dark-skinned people among which it accounts for 50% of melanomas. Its appearance does not seem to be associated to sun exposure. ALM is sometimes referred to as "hidden melanoma" because it often appears on part of the body not easily examined or not thought to be examined such as palms, soles, mucous membranes and underneath or near fingernails and toenails. ALM usually begins as an irregular shaped tan, brown or black spot; as ALM increases in size, it usually becomes more irregular in shape and colour and the tumour invades deeply into the skin, even if the surface of the lesion remains flat. The median age of occurrence is 65 years, with an equal sex distribution (Lens, 2008).

❖ Non-pigmented subtypes of melanoma

While uncommon, melanoma also exits without brown or black pigmentation. An uncommon subtype is called amelanotic melanoma that usually appears as a pink or red nodule. Desmoplastic melanoma (DM) is a rare melanoma that mostly arises on sun-exposed skin of the head and neck of people aged 60 or more. Myxoid melanoma (MM) is an uncommon melanoma variants that is characterized by an important myxoid change that can be seen in primary melanomas, recurrences and metastases even when absent in the primary tumour (Lens, 2008).

1.2.3.2 Pathological staging of melanoma

In 2001 the American Joint Committee on Cancer (AJCC) Tumour-Nodes-Metastasis (TNM) staging classification incorporated Breslow depth, Clark's level, ulceration and pathological micro-staging attributes. The TNM classification is widely used to describe many human cancers. In general T refers to the primary tumour size, N describes the involvement of regional lymph nodes, and M indicates the presence or absence of distant metastases. Paralleling the staging system for other cancers, melanoma patients with localized disease are characterized as stage I or II, those with regional metastases are considered stage III, and those with distant metastases are considered IV. To characterize the primary tumour, T in melanoma uses Breslow Depth (Breslow, 1970), Clark's level (Clark et al, 1969) and the presence or absence of ulceration.

Breslow depth is measured in millimeters and can be divided in T1 (<1.0mm), T2 (1.01 to 2.0mm), T3 (2.01 to 4.0mm), T4 (>4.0mm). Clark's level describes the depth of invasion and is useful for prognostic differentiation of T1 lesions. Level I is limited to the epidermis (in situ melanoma); Level II reaches the papillary dermis; Level III fills the papillary dermis; Level IV involves the reticular dermis; Level V penetrates within the subcutaneous fat. Tumours that have a Breslow depth of less than 1mm and have a Clark's level of II or III without ulceration remain T1a melanomas; T1b melanomas are ulcerated or have a Clark's level IV or V. A final version of the 2009 AJCC staging and classification of melanoma included the mitotic index as a prognostic factor in stratifying T1 melanoma. In addition, the Clark level is not included as a parameter of staging since is not any longer an independent prognostic factor when mitotic index is included in the analysis.

Proliferation of the primary melanomas, defined by the mitotic index, is a powerful and independent predictor of survival. As a result, primary tumour mitotic rate is now a required element for the melanoma staging system. This prognostic value is maintained when age, sex, location of the primary tumour, thickness and ulceration are introduced in the multivariate model.

The N category considers the status of regional lymph nodes. Stages I or II do not include any nodal or metastatic involvement. For Stage III melanoma, the number and dimension (micrometastasis versus macrometastasis) of nodal metastases or the presence of in-transit metastases (lesions that reside more than 5 cm from the primary tumour) or satellite lesions (lesions that reside within 5 cm from the primary tumour) are the most significant predictors of outcomes (Table 1.9).

Distant metastases characterize Stage IV melanomas and can occur in skin and subcutaneous tissues, distant lymph nodes (M1a), lung (M1b), and other visceral organs (M1c).

Approximately 81% of cutaneous melanomas are locally confined; 12% are diagnosed after a regional spreading; 4% are diagnosed with distant metastases and for the remaining 4% the staging is unknown. In the absence of nodal or distant metastases, tumour thickness and presence or absence of ulceration remain the best predictors of survival outcome (Balch et al, 2009). Presence of ulceration signifies a greater risk for metastases and thus the survival rate for patients with a given T category with ulceration is nearly the same as patients of the next T category without ulceration; moreover ulceration upstages melanoma and confers poorer prognosis when melanoma is locally confined.

Patients with an unidentifiable primary tumour location account for 2-6% of all melanoma patients. Among this group, patients with subcutaneous or in-transit metastases show a 5-year survival of 83%; 50% in individuals with lymph node metastases and a median survival of 6 months for patients with disseminated disease (Schlagenhauff et al, 1997). (Figure 1.9)

Figure 1.9. Melanoma survival. AJCC TNM stages and overall survival of patients with cutaneous melanoma. (Adapted from Balch et al., 2009).

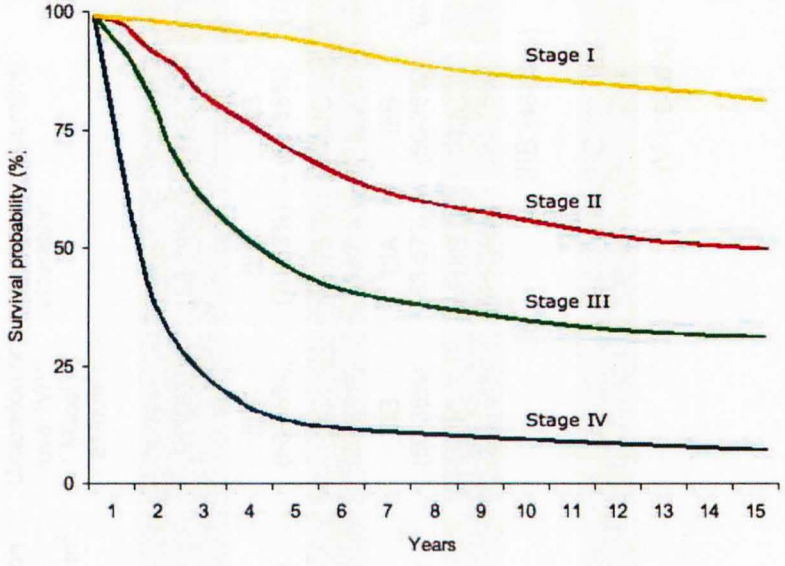


***Table 1.9**: Integrated TNM staging. M: metastasis; Ulc: ulceration; mM: micrometastasis; MM; macrometastasis; trM: in transit metastasis; sM satellite metastasis. Percentage indicated the 5-and 10-year survival rates. Adapted from Cho and Chiang, 2010 and Balch et al, 2009.*

		Tis	**T1** (<0,1mm)		**T2** (1.01-2.0mm)		**T3** (2.01-4.0mm)		**T4** (>4.0mm)	
			a No ulceration level II/III Mitosis<1/mm^2	b Ulceration or level IV/V Mitosi ≥1/mm^2	a no ulceration	b ulceration	a no ulceration	b ulceration	A no ulceration	b ulceration
Nodes	N0	0	IA (95-88%)	IB (91%83)	IB (89-79%)	IIA (77-64%)	IIA (79-64%)	IIB (63-51%)	IIB (67-54%)	IIC (45-32%)
	N1a (1 n, mM)		IIIA (70-63%)	IIIB (53-38%)	IIIA (70-63%)	IIIB (53-38%)	IIIA (70-63%)	IIIB (53-38%)	IIIA (70-63%)	IIIB (53-38%)
	N1b (1 n, MM)		IIIB (59-48%)	IIIC (29-24%)	IIIB (59-48%)	IIIC (29-24%)	IIIB (59-48%)	IIIC (29-24%)	IIIB (59-48%)	IIIC (29-24%)
	N2a (2-3 n, mM)		IIIA (63-57%)	IIIB (50-36%)	IIIA (63-57%)	IIIB (50-36%)	IIIA (63-57%)	IIIB (50-36%)	IIIA (63-57%)	IIIB (50-36%)
	N2b (2-3 n MM)		IIIB (46-39%)	IIIC (24-15%)	IIIB (46-39%)	IIIC (24-15%)	IIIB (46-39%)	IIIC (24-15%)	IIIB (46-39%)	IIIC (24-15%)
	N2c (0 n, trM/sM)					IIIB (46-39%)				
	N3 (>4 n, trM/sM)					IIIC (27-18%)				
Metastases (M)	M1a (Distant skin, subcutaneous, or n M), normal LDH.					IV (19-16%)				
	M1b (lung M, nomal LDH)					IV (7-3%)				
	M1c (visceral M other then lung, LDH normal; or any distant metastasis with elevated LDH)					IV (10-6 %)				

1.2.4 Biology of melanoma: molecular mechanisms of melanocytic transformation and melanoma progression.

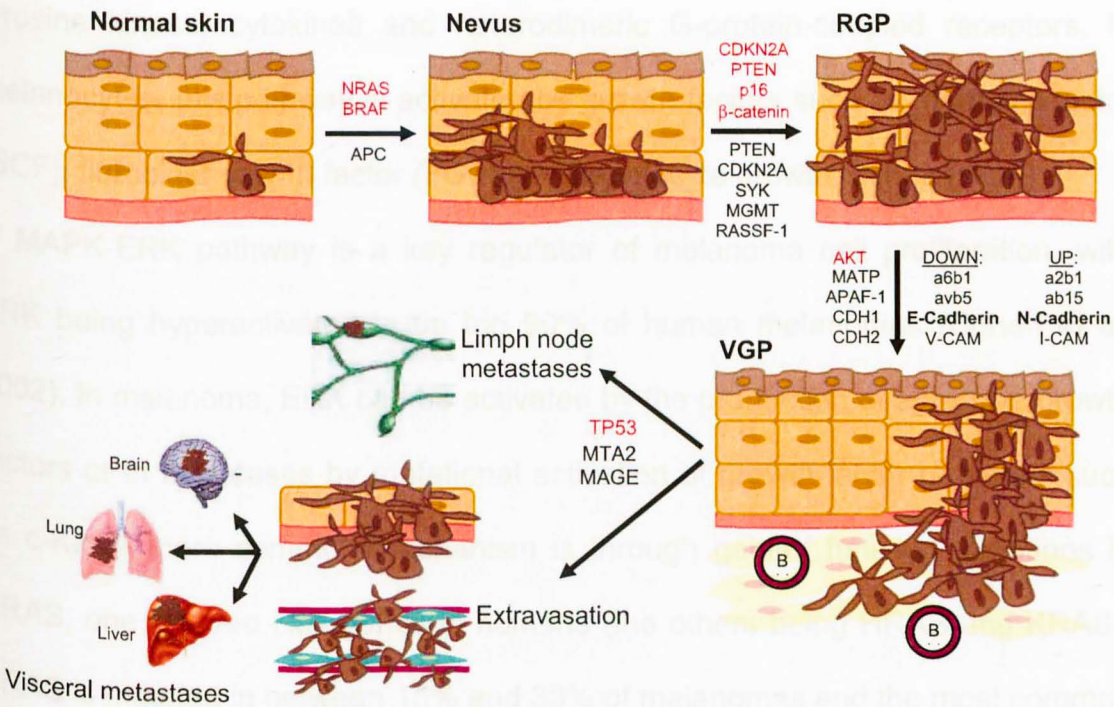
Melanoma is a complex genetic disease, whose management will require an in-depth understanding of the biology underlying its initiation and progression. Until recently, melanomas were thought to arise from normal melanocytes undergoing malignant conversion caused by the sequential accumulation of genetic and molecular alteration. Cutaneous melanocytes originate from highly motile neural-crest progenitors that migrate to the skin during embryonic development (Slominski et al, 2004). In the skin, melanocytes reside in the hair follicles and in the basal layer of epidermis. Melanocyte survival, differentiation, proliferation and motility are regulated by keratinocytes.

Although the precise mechanisms underlying melanoma development are not completely clarified, recent studies have provided a better understanding of melanoma biology. Mutations in critical regulatory genes, the production of autocrine growth factors and the loss of adhesion receptors all contribute to disrupted intracellular signaling in melanocytes, allowing them to escape their regulation by keratinocytes (Haass et al., 2004). Consequently, melanocytes can proliferate and spread, leading to formation of a nevus. Melanocytes proliferation can be restricted to the epidermis (junctional nevus), the dermis (dermal nevus) or overlapping components of both (compound nevus). Common nevi are generally benign but they can progress to the radial-growth-phase (RGP) melanoma, an intra-epidermal lesion that can involve some local microinvasion of

the dermis. RGP cells can progress to the vertical-growth-phase (VGP), a more dangerous stage in which the cells have metastatic potential, with nodules or nests of cells invading the dermis. Not all melanomas pass through each of these individual phases; RGP and VGP can both develop directly from isolated melanocytes or nevi, and both can progress directly to metastatic malignant melanoma. Of note, RGP melanoma cells are mainly confined to the epidermis, are not tumorigenic in animal models and unable to metastasize in patients. In contrast, VGP melanomas deeply invade the skin, are highly proliferative and tumorigenic in experimental models and rapidly metastasize in patients (Hsu et al, 2002) (Figure 1.10).

By the usage of several genetic approaches, that included mutation analysis, some crucial cell-signaling pathways activated in this disease have been identified and they are described below.

Figure 1.10. Scheme of melanoma progression. Aberrant proliferation of normal melanocytes, results in the formation of benign or dysplastic nevi. Radial growth phase (RGP) melanoma exhibits the ability to grow intraepidermally, followed by invasion of the dermis in the vertical growth phase (VGP), and culminating with lymphatic invasion or extravasation leading respectively to lymph node or visceral metastasis. Progression from a radial growing phase (RGP) to vertical growing phase (VGP) melanoma is associated with profound changes in expression of adhesion receptors. In particular there is an upregulation of N-cadherin, integrin αVβ3 and ICAM-1 expression and a downregulation of E-cadherin and integrin α6β1. Spontaneous DNA mutations (black) have been observed in several genes and are postulated to be involved at different stages of melanoma progression. Recent studies have also provided evidence for the presence of several genes whose expression is altered in melanoma by epigenetic modulations (Red). B: blood vessels. (Adapted from Zaidi et al., 2008).



1.2.4.1 Proliferative pathways

The MAPK-ERK pathway (including the cascade of NRAS, BRAF, MEK1/2, and ERK1/2 proteins) is one of the major signaling cascade involved in the control of cell growth, proliferation and migration (Davies et al, 2002). The RAS/Raf/MEK/ERK pathway regulates cell fate decision downstream of receptor tyrosine kinase, cytokines and heterodimeric G-protein-coupled receptors. In melanocytes, this pathway is activated by growth factors such as stem-cell factor (SCF), fibroblast growth factor (FGF) and hepatocyte growth factors (HGF).

MAPK-ERK pathway is a key regulator of melanoma cell proliferation, with ERK being hyperactivated in up top 90% of human melanomas (Cohen et al, 2002). In melanoma, ERK can be activated by the production of autocrine growth factors or in rare cases by mutational activation of growth-factor receptors such as c-Kit. A more common mechanism is through gain-of function mutations in NRAS, one of three Ras genes in humans (the others being HRAS and KRAS). NRAS is mutated in between 15% and 30% of melanomas and the most common substitution is leucine for glutamine at position 61 (Q61L) (Table 1.10).

However, in melanoma, the most commonly mutated component of this pathway is BRAF, BRAF is mutated in 50% to 70% of melanomas (Table 1.10), and the most common mutation is a glutamic acid for valine substitution at position 600 (V600E) (Davies et al, 2002). ^{V600E}BRAF supports constitutive ERK signaling, thus promoting tumour proliferation and survival. ^{V600E}BRAF also contributes to neoangiogenesis by stimulating autocrine secretion of vascular

endothelial growth factor (VEGF). Recent studies have identified several genes in melanoma that function downstream of ^{V600E}BRAF such as BRN-2 (POU domain class 3 transcription factor), the cell cycle regulators cyclin D1 and p16INK4a, the tumour maintenance enzymes matrix metalloproteinase-1 and inducible nitric oxide synthase. Activating BRAF mutations have been detected in melanoma patients only at the somatic level and in common cutaneous nevi. The presence of BRAF mutation in nevi strongly suggests that BRAF activation is necessary but not sufficient for the development of melanoma.

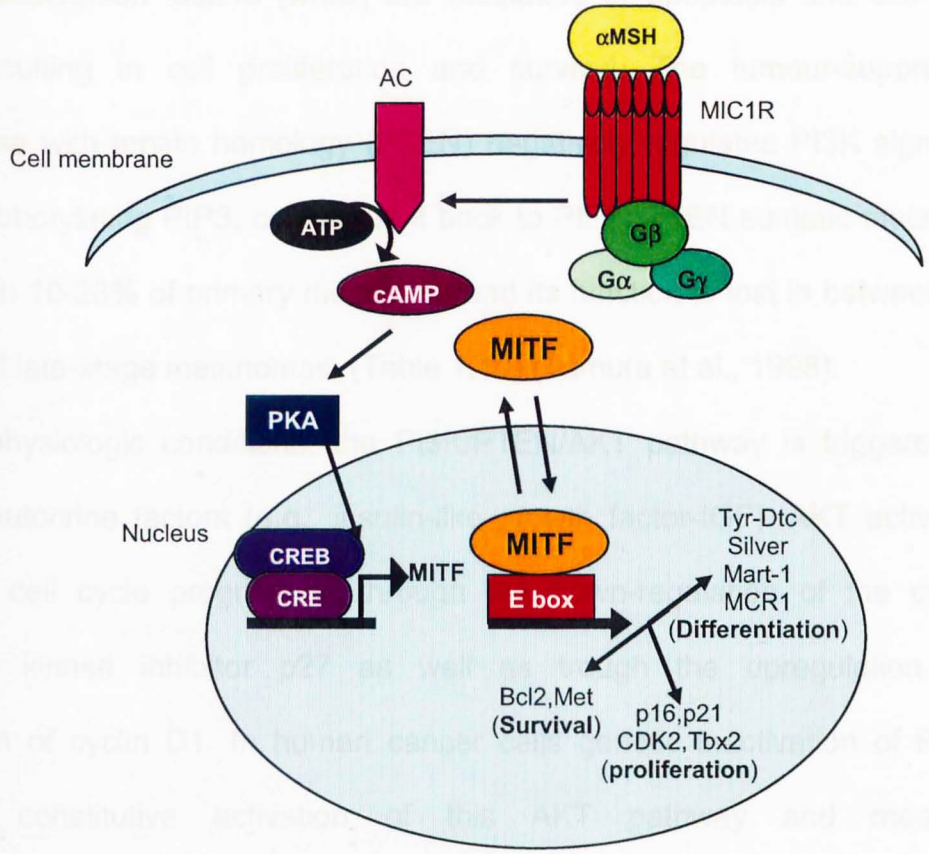
Table 1.10 Selected genetic alteration in malignant melanomas (Adapted from Gray-Schopfer et al., 2007).

Gene Type	Gene	Chromosomal location	Alteration frequency in melanoma (%)	Type(s) of alteration
Oncogenes	BRAF	7q34	50-70%	Mutated
	NRAS	1p13.2	15-30%	Mutated
Tumour suppressor	CDKN2A	9p21	30-70%	Deleted, mutated, silenced
	PTEN	10q23.3	5-20%	Deleted or mutated
	APAF-1	12q23.1	40%	Silenced
	p53	17q21	10%	Lost or mutated
Others	CylinD1	11q13	6-44%	Amplified
	MITF	3p14.1-p12.3	15.20%	Amplified

The different regulatory functions of MITF are associated to the level of expression of the MITF protein. In particular, high level of MITF protein exerts an anti-proliferative activity in melanoma cells. High levels predispose to cell cycle arrest and differentiation, presumably by controlling cell cycle regulators (such as p16INK4a, cyclin-dependent kinase 2 (Cdk2) and $p21^{Cip1}$, whereas critically low levels lead to cell cycle arrest and apoptosis; only intermediate level favors proliferation. The presence of oncogenic BRAF and low level of MITF protein

were found in invasive melanoma cells and have been associated with poor prognosis and clinical disease progression. Of note, MITF resides downstream of the ERK and the PI3K pathways, suggesting that MITF could integrate extracellular pro-survival signals. In particular, MITF is targeted for degradation after its phosphorylation by ERK. Indeed, constitutive activation of ERK by ^{V600E}BRAF results in constant down-regulation of MITF. It has recently been shown that MITF is amplified in a small percentage (10–16%) of metastatic melanomas in which BRAF is mutated (Table 1.10) (Gray-Schopfer et al. 2007) (Figure 1.11).

Figure 1.11 MITF signaling pathway. MITF is master regulator of melanocyte-specific transcription. Several pathways affect the level of MITF expression by acting on the MITF promoter. Specifically, α-MSH binds to the G protein–coupled MC1R, which acts through adenylate cyclase (AC) to stimulate binding of CREB1 to the MITF promoter thus inducing the transcription of MITF target genes: genes involved in melanin production (*TYR*, *TRP1*, and *DCT*), genes important for survival (*BCL2*), and numerous melanoma markers (Mart-1, MCR1, and Silver). (Adapted from Sekulic et al., 2008).

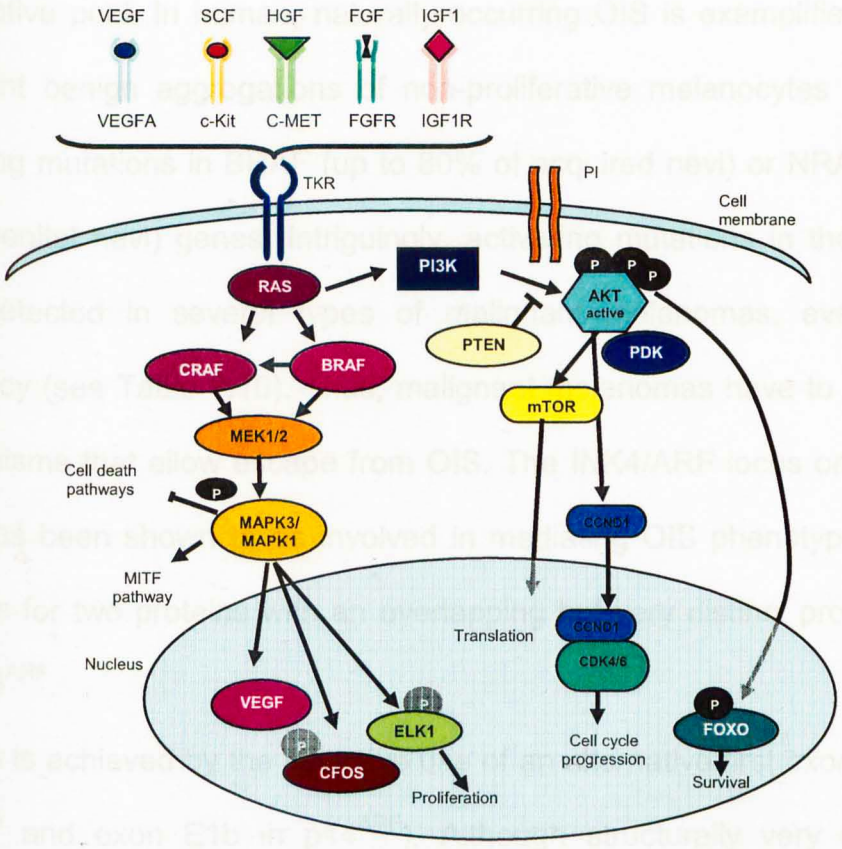


Another signaling pathway that has emerged as important in melanoma is the phosphoinositide-3-OH kinase-AKT (PI(3)K) pathway (Figure 1.12). Activated lipid kinase phosphoinosite-3-kinase (PI(3)Ks) converts through hyper-phosphorylation, the lipid second messenger phosphatidylinositol (4,5) bisphosphate (PIP2) into phosphatidylinositol (3,4,5) triphosphate (PIP3), which recruits and activates phosphatidylinositol-dependent kinase 1 (PDK1). PDK1 in turn phosphorylates and activates AKT (also known as protein kinase B (PKB),

The AKT protein is a serine/threonine kinase and belongs to the AKT protein kinase family (AKT1, AKT2, and AKT3) that is overexpressed in up to 60% of melanomas (Stahl et al., 2004). AKT inhibits the activities of the forkehead (FOXO) transcription factors (which are mediators of apoptosis and cell-cycle arrest), resulting in cell proliferation and survival. The tumour-suppressor phosphatase with tensin homology (PTEN) negatively regulates PI3K signaling by dephosphorylating PIP3, converting it back to PIP2. PTEN somatic mutations are found in 10-20% of primary melanoma and its function is lost in between 5% and 20% of late-stage melanomas (Table 1.10) (Tamura et al., 1998).

Under physiologic conditions, the PI3K/PTEN/AKT pathway is triggered by paracrine/autocrine factors (e.g., insulin-like growth factor-IGF). AKT activation stimulates cell cycle progression through the down-regulation of the cyclin-dependent kinase inhibitor p27 as well as trough the upregulation and stabilization of cyclin D1. In human cancer cells genetic inactivation of PTEN leads to constitutive activation of this AKT pathway and mediates tumourigenesis. Notably, in melanoma, NRAS and BRAF mutations are mutually exclusive, as are NRAS and PTEN mutations, whereas BRAF and PTEN mutations are coincident in about 20% of cases (Tsao et al, 2004) (Figure 1.12).

Figure 1.12 Crucial pathways involved in melanoma progression. Within the MAPK pathway, activated receptors lead to SHC-mediated activation of RAS and propagation of signaling through RAF, MEK (also known as MAP2K), and MAPK (also known as ERK). Activated MAPK transduces signals that regulate multiple cell processes including proliferation, differentiation, angiogenesis, and survival. The second essential signaling pathway is the PI3K/AKT pathway. PI3K, a lipid kinase, catalyzes the phosphorylation of phosphatidylinositol (PI). Triple phosphorylation of PI is necessary for the association of AKT with the cell membrane and its subsequent activation by PDK1. The key regulatory component is PTEN, a phosphatase that regulates PI3K and SHC phosphorylation. RAS may also regulate PI3K activity. Through activated AKT, several processes are activated including inhibition of apoptosis, survival gene transcription, cell cycle progression, protein translation, and cell growth and proliferation. The balance between AKT activation and PTEN regulatory activity is a key determinant in cell cycle progression. FGF = fibroblast growth factor; HGF = hepatocyte growth factor; IGF = insulin like growth factor; P = phosphate; VEGF = vascular endothelial growth factor. (Adapted from Sekulic et al., 2008).



1.2.4.2 Senescence and apoptotic pathways

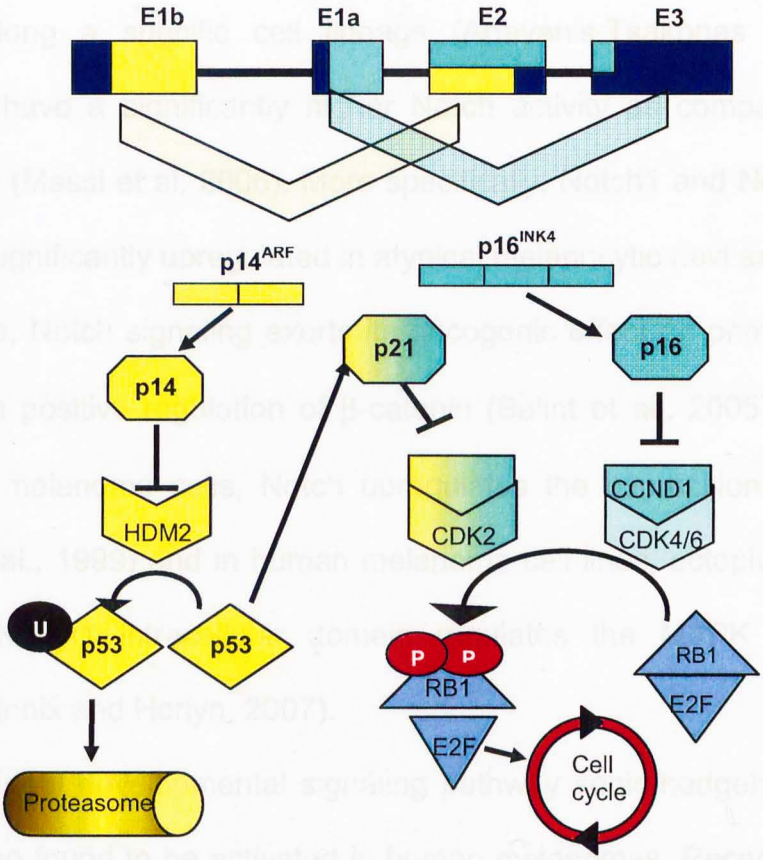
Senescence is a form of irreversible arrest of cell proliferation. In normal cells it can occur due to telomere shortening (replicative senescence), oncogenic activation (oncogene-induced senescence, OIS), and/or cellular stress due to intense proliferative signals. OIS is emerging as a potent cancer-protective response to oncogenic events, serving to eliminate early neoplastic cells from the proliferative pool. In human, naturally occurring OIS is exemplified by nevi that represent benign aggregations of non-proliferative melanocytes often carrying activating mutations in BRAF (up to 80% of acquired nevi) or NRAS (up to 80% of congenital nevi) genes. Intriguingly, activating mutations in the same genes were detected in several types of malignant melanomas, even if at lover frequency (see Table 1.10). Thus, malignant melanomas have to develop some mechanisms that allow escape from OIS. The INK4/ARF locus on chromosome 9p21 has been shown to be involved in mediating OIS phenotypes. This locus encodes for two proteins with an overlapping but very distinct proteins: $p16^{INK4a}$ and $p14^{ARF}$.

This is achieved by the selective use of an alternative first exon (exon E1a in $p16^{INK4a}$ and exon E1b in $p14^{ARF}$). Although structurally very different, both protein products act as negative regulators of cell cycle progression. The tumour suppressor protein p16INK4a binds the cyclin-D-dependent protein kinases CDK4 and 6, blocks their activity thus preventing Rb phosphorylation and leading to cell cycle arrest (Serrano et al, 1993). In contrast, $p14^{ARF}$ regulates tumour

protein 53 (p53) activities by inhibiting HDM2. HDM2 is an ubiquitin ligase that targets p53 for degradation by the proteasome. High levels of $p14^{ARF}$ stabilize p53. The p53 protein arrests cell division at the G1 phase to allow DNA repair or to induce apoptosis of potentially transformed cells. Impairment of the $p14^{ARF}$–HDM2-p53 cascade, whose final effectors are the bax/bcl2 proteins, results in an accumulation of genetic damage in cells which promote tumour formation (Figure 1.13). In human melanoma, CDKN2A inactivation by deletion, mutation or promoter silencing (through hypermethylation) generally affects p16INK4a alone or $p16^{INK4a}$ and $p14^{ARF}$ simultaneously and its lost could be one possible mechanism overriding RAF-RAS induced senescence. An additional alternative mechanism suppressing senescence relies on C-MYC. C -MYC is a transcription factor frequently upregulated in human malignancies including metastatic melanoma (Bansal and Nikiforov, 2010) that regulates the expression of multiple genes involved in many cellular processes including promotion of proliferation. The observation that senescence can be induced via autocrine/paracrine pathways has been confirmed by the finding that $BRAF^{V600E}$ activates an inflammation-specific transcriptome in normal human melanocytes. Furthermore, some cytokine and chemokine expression declined during OIS bypass. In particular, some experiments revealed a causal relationship between the induction of IL-6 and IL-8 and the ability of cells to undergo OIS. In response to oncogenic stress, IL6 and IL8 genes were activated by the transcription factor C/EBPβ, upon its recruitment to the promoter. C/EBPβ depletion enables cells to effectively bypass OIS. Escape of OIS is correlated with the lost of expression of

both cytokines. Since IL-6 depletion was followed by a strong decline in the levels of both C/EBPβ and IL-8, IL-6 could act as a positive feedback network regulating OIS (Kuliman et al., 2008). These findings are supported by the positive correlation between increased IL-8 expression and p16INK4A positivity in growth arrested cells in human colorectal carcinoma thus sustaining a connection between senescence, inflammation and inflammation.

Figure 1.13 CDKNA pathways. The *CDKN2A* locus on chromosome 9p21 has an unusual structure because it encodes for 2 overlapping proteins: $p16^{INK4A}$ and $p14^{ARF}$ using an alternative first exon (exon E1a in $p16^{INK4A}$ and exon E1b in $p14^{ARF}$). The $p16^{INK4A}$ protein inhibits the activation of CDK4 and CDK6 by cyclinD1 (CCND1), thereby preventing the subsequent phosphorylation of RB1. Underphosphorylated RB1 sequesters the transcription factor E2F and prevents them from inducing the progression from G1 to S phase of the cell cycle In contrast, $p14^{ARF}$ regulates tumour protein 53 (p53) activity by inhibiting MDM2, a ubiquitin ligase that otherwise targets p53 for degradation by theproteasome. High levels of $p14^{ARF}$ stabilize p53 permitting it to induce p21. In the absence of functional ^{p14ARF,} uncontrolled ubiquitination and degradation of p53 removes this important cell cycle brake, leading ultimately to hyperphosphorylation of RB1 and cell cycle progression. P = phosphate; U= ubiquitin. (Adapted from Sekulic et al., 2008).



1.2.4.3 Novel signaling pathways involved in melanoma biology

Notch proteins are a family of single-pass transmembrane proteins (Notch 1-4) that act as membrane receptors activated by specific transmembrane ligands through a direct cell-cell interaction. The Notch signaling pathway plays a pivotal role in self-renewal of adult stem cells, as well as in the differentiation of precursor along a specific cell lineage (Artavanis-Tsakonas et al., 1999). Melanomas have a significantly higher Notch activity as compared to normal melanocytes (Massi et al, 2006). More specifically, Notch1 and Notch2 and their ligands are significantly upregulated in atypical melanocytic nevi and melanomas. In melanoma, Notch signaling exerts its oncogenic effect on primary melanoma cells through positive regulation of β-catenin (Balint et al., 2005). Moreover, in early-phase melanoma cells, Notch upregulates the expression of N-cadherin (Sanders et al., 1999) and in human melanoma cell lines, ectopic expression of the active Notch1 intracellular domain regulates the MAPK and PI3K-Akt pathways (Pinnix and Herlyn, 2007).

The additional developmental signaling pathway sonic-hedgehog (SHH)-GLI, has been also found to be activated in human melanomas. Recently it has been shown that SHH-GLI signaling is active in human hair follicles and required for normal *in vitro* proliferation of melanocytes (Rittié et al., 2009). SHH-GLI is also involved in proliferation and survival of melanoma as evidenced both *in vitro* and *in vivo* in melanoma xenografts (Stecca et al., 2007). More recently an association between GLI2 and melanoma invasion and metastasis has been

described in a series of human melanoma cell lines by *in vitro* and *in vivo* assays (Alexaki et al., 2010). The molecular aspects of these pathways as well as their involvement in stem cell biology have been discussed in the section 1.1.2.4.1.

1.2.4.4 Melanocyte development and melanoma

Melanocytes are specialized melanin producing cells, and are responsible for skin, hair and eye pigmentation in vertebrate organisms. Melanocytes synthesize pigment melanin within special organelles termed melanosomes. Once synthesized, melanin is deposited in melanosomes to form melanin granules. These melanin granules are transferred from melanocytes through their dendrites to adjacent keratinocytes, where melanin is accumulated to generate pigmented skin or hairs. The principal function of melanocytes is to protect skin from genotoxic stress of UV radiation but melanocytes also play an essential role in the hearing system.

Melanocytes are derived from a group of highly pigmented migratory embryonic cells called neural crest cells. The neural crest is induced at the time of gastrulation, in the zone between the neural and non-neural ectoderm and it gives rise to a number of cell types, including osteocytes, chondrocytes and sensory neurons. Cell labeling studies indicate that neural crest cells are initially multipotent but gradually become lineage-restricted in development potential (Dorsky et al., 1998). In most cases, this potential is determined by its anatomic location with melanocytes originating from the trunk neural crest together with

adrenal cells and sensory ganglia. The development of a multipotent neural crest stem cell into mature melanocytes involves a bipotent glial-melanocyte lineage progenitor that develops into unpigmented precursor cells called the melanoblasts (Dupin et al., 2000). Early neural crest induction depends in part on intact BMP signaling together with a pivotal role of Snail/Slug family of transcription factors that stimulate neural crest progenitor cells to undergo an epithelial-mesenchymal transition (EMT) (Cano et al., 2000). The Notch family of proteins plays an essential role in fate decision of early progenitors preventing cells in the premigratory neural crest from taking on a neural fate and instead becoming unspecified neural crest precursors (Kelsh et el., 2006). Commitment to the melanoblast stage usually occurs in the neural tube or after migration has started. Wnt signaling is important in promoting a cell fate decision towards melanoblasts and mature melanocytes as suggested by the study in Wnt deficient mice (Ikeya et al, 1997). However, recent data suggest the existence of an additional distinct origin of melanoblasts. According to these new findings, skin melanoblasts originate from Schwann cell precursors that are positioned along the developing nerves (Adameyko et al, 2009).

Once melanoblasts arise, they migrate along the dorsolateral pathway and in mammals most of the melanoblasts further invade the overlying epidermis, where they proliferate and migrate extensively to distribute throughout the entire epidermis. The number of melanocytes in the epidermis is tightly controlled and this is primarily due to their interaction with local keratinocytes. A major homeostatic factor in the adult skin is Stem Cell Factor (SCF). Epidermal

keratinocytes produce SCF in both a membrane bound and soluble form and SCF stimulation or inhibition has been shown to drive proliferation or loss, respectively, of melanocytes in human skin (Grichnik et al., 1998).

In the hair follicle, melanoblasts are segregated into two populations: the hair matrix melanocytes, which are responsible for pigmentation of the initial hairs; while the second population consists of melanocyte stem cells, which are localized at the lower permanent portion of the hair follicle (the bulge region) and are responsible for the maintenance of the hair follicle pigment system in the subsequent hair cycles (Nishimura et al., 2002). Hair growth occurs in alternating phases of proliferation (anagen), regression (catagen) and quiescence (telogen). Melanoblasts appear at the onset of anagen phase in the hair matrix where they actively proliferate and differentiate into mature melanocytes. During catagen, these melanocytes are depleted from the follicles by apoptosis, and finally melanocytes become absent in telogen hair follicle until the next melanogenesis initiated in the subsequent anagen phase. It is thought that at least one melanocyte stem cell remains in the bulge region during the entire hair cycle, ensuring the production of melanocytes at the beginning of a new cycle while transit amplifying melanocytes go out of the niche and migrate to the epidermis, were they can further differentiate into pigmented melanocytes.

In non hair region of the human skin, melanoblasts stay immature and reside on the basement membrane of the interfollicular epidermis where they play a role in formation of skin pigmentation (Osawa et al., 2005). Whereas the origin of the human epidermal melanocytes has not been determined yet, it has been

hypothesized that these melanocytes may also arise from the bulge melanoblasts in the hair follicle as suggested by the observation that repigmentation process of Vitiligo, a condition where the epidermal melanocytes are lost from the skin, is initiated in a perifollicular manner (Falabella and Barona, 2009). Hence, these observations indirectly provide evidence for a role of human follicles as reservoir also for the epidermal melanocytes.

Furthermore, using *in vitro* culture conditions suitable for human embryonic stem cells, multipotent adult stem cells have been recently isolated from human hair follicles. These cells do not express melanocytic markers, but express neural crest and neural stem cell markers as well as the embryonic stem cell transcription factors Nanog and Oct4. These precursor cells are self-renewing and multipotent *in vitro* acquiring lineage differentiation markers under specific culture conditions and they also demonstrate appropriate functions in *ex vivo* conditions (Yu et al, 2006).

Through the extensive studies of mutant animals, a number of key genes for melanoblast development have been identified: examples of these genes include Pax3 (paired Box 3), Sox10 (Sex-determining region Y-box 10), MITF (microphtalmia-associated transcription factors), Edn3 (endothelin 3), Ednrb (endothelin receptor B), kit (c-kit tyrosine kinase receptor), KitL (kit ligand, also called as SCF or steel factor) and Snai2 (also called Slug) (Bennett and Lamoreux, 2003; Hou and Pavan 2008).

Pax3 plays a key role in neural crest development as well as muscle and cardio-vasculature formation. Mice with loss of functional Pax3 exhibit a white

spotting phenotype suggesting a role of Pax3 in melanoblasts development (Moase and Trasler, 1992).

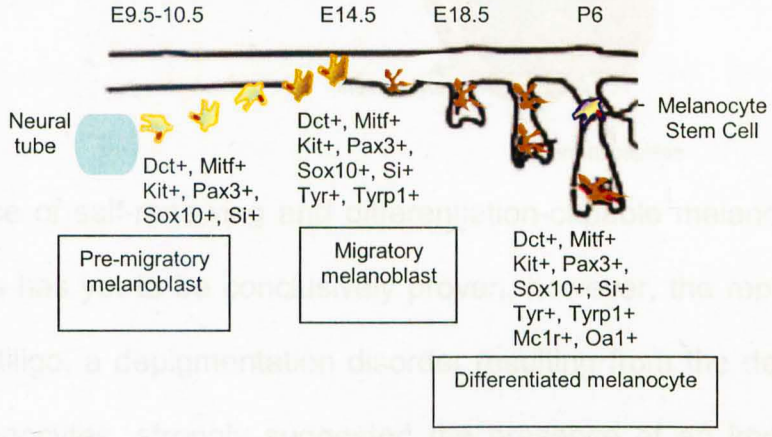
MITF is a melanocyte lineage-specific gene that encodes a transcription factor particularly important in melanocyte development. MITF regulates the melanocyte lineage by activating several pigment-producing genes, such as tyrosinase-related protein-2 or TRP-2 and tyrosinase and also melanoblasts survival depends on MITF (Hornyak et al., 2001). A direct influence of Pax3 in antagonizing Mitf signaling has been demonstrated, suggesting a possible role of Pax3 in maintaining a balance between undifferentiated and differentiated status of melanoblasts (Lang et al., 2005).

Sox10 is expressed in pre-migratory neural crest cells and its expression is gradually restricted in glial and melanocyte lineages (Hou and Pavan 2008) with an indispensable role in promoting survival, migration and differentiation of melanocytes. The downstream targets of Sox10 are not completely clarified, but Sox10 preferentially enhances expression of MITF and tyrosinase (Murisier and Beermann, 2006). Endothelin signals are essential for survival and migration of melanocytes in their early stage of development (Lee et al., 2003).

The type III receptor tyrosine kinase c-Kit is essential for melanocyte development. It is involved in the molecular process of pigmentation. In fact humans heterozygous for a mutation in the c-KIT gene have a pigmentation disorder called piebaldism and mouse mutants for the c-kit gene or its ligand steel/stem cell factor, also demonstrate varying degrees of pigmentation abnormalities.

Molecularly, melanocyte stem cells can be distinguished from their more differentiated progeny by a Pax3+, Tyr-, Tyrp1-, Kit-, MITF-, Sox10- phenotype (Osawa et al., 2005). Expression or absence of Pax3, Sox10 and MITF has been shown to be critical for melanocyte SC maintenance, differentiation and quiescence. Importantly, elevated levels of Wnt signaling molecules antagonize the regulatory balance of Pax3, Sox10 and MITF towards terminal differentiation (Nishikawa and Osawa, 2007) (Figure 1.14).

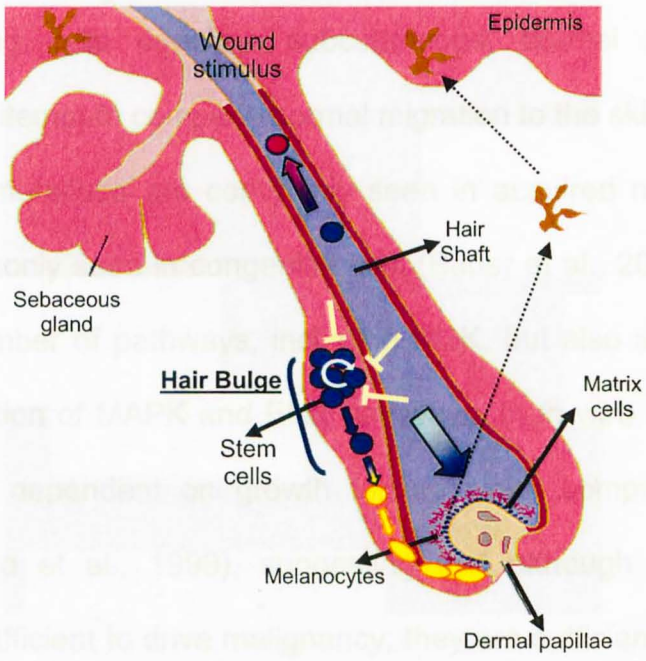
Figure 1.14 Scheme of melanocyte development. During mouse embryogenesis, melanoblasts emerge in the neural crest at embryonic day (E) 9.5–10.5. These earliest stages of melanoblasts are characterized by the expression of Dct, Kit, Mitf, Pax3, Sox10, and Si. After arising from neural crest, melanoblasts migrate to the developing dermis. Then, at E12.5–13.5, they enter the epidermis where they actively migrate and proliferate to be distributed over the entire body. In addition to the pre-migratory melanoblast markers, these migratory melanoblasts express several melanosomal proteins including Tyr and Tyrp. Upon initiation of hair follicle morphogenesis, the melanoblasts enter the newly developing hair and are segregated into two populations. In the hair matrix, melanoblasts differentiate into mature melanocytes and acquire the expression of Mc1r and Oa. In the bulge region melanoblasts are restricted in a resting status to become melanocyte stem cells gradually downregualting several melanoblast markers including Kit, Mitf, Pax3, Sox10, Tyr, andTyrp1 (Adapted from Osawa et al., 2005).



Melanocyte stem cells appear to be maintained in a quiescent status in the niche and the fact that melanocyte stem cells are randomly scattered within the

bulge region without forming into cell clusters led to the hypothesis that the bulge region itself serves as a niche for the melanocyte stem cells (Figure 1.15).

Figure 1.15 Localization of the melanocytes stem cells from the hair follicle. The hair follicle is divided into two portions: a transit portion that completely reforms itself over the hair cycle; and a permanent portion of the follicle that is maintained throughout hair cycling that includes the hair bulge. The bulge is thought to contain melanocyte stem cells that are responsible for the formation of the new hair follicles during hair cycling and repair the epidermis upon injury. The bulge acts as a specialized niche, surrounded by other cell types, which together provide cues that maintain melanocytes stem cells in an undifferentiated state. The hair follicle melanocytes reside in the lower part of the hair bulb among the matrix keratinocytes. The matrix is in the deepest portion of the follicle that envelops the dermal papilla. (Adapted from Hoffman 2000; Fuchs 2007).



The existence of self-renewing and differentiation-capable melanocytes stem cells in humans has yet to be conclusively proven, however, the repigmentation processes in vitiligo, a depigmentation disorder resulting from the destruction of functional melanocytes, strongly suggested the presence of an immature pool that replenishes the skin with functional melanocytes upon disease control (Yu, 2002). Moreover, different melanocyte phenotypes are distinguishable in the

human epidermis and hair follicle, including amelanotic, non-proliferative cell types, and pigmented more proliferative melanocytes (Grichnik et al., 1996).

1.2.4.4.1 Origin of melanocytic nevi

Nevi are benign clonal proliferations of cells expressing the melanocytic phenotype. In congenital nevi, it is presumed that a precursor cell is mutated and that by uncontrolled proliferation it gives rise to an excessive number of daughter cells. The migrating cells populate subcutaneous, dermal and epidermal structures, as they attempt to complete normal migration to the skin surface.

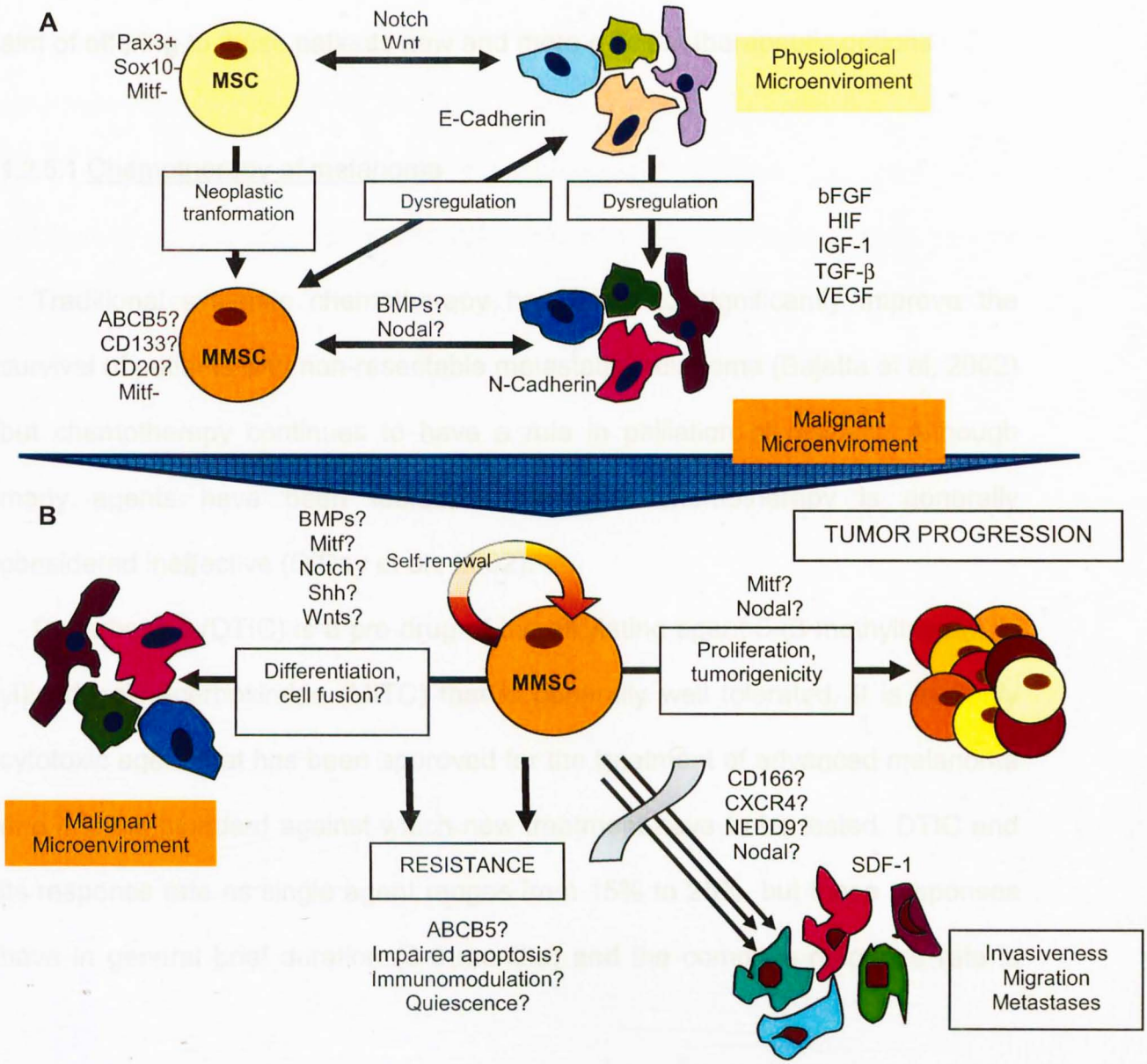
B-Raf-activating mutations are commonly seen in acquired nevi and N-Ras mutations are commonly seen in congenital nevi (Bauer et al., 2007). N-Ras not only activates a number of pathways, including PI3K, but also activates B-Raf, leading to an activation of MAPK and ERK pathways. In *in vitro* cultures, nevus cells are also less dependent on growth factors when compared to normal melanocytes (Alanko et al., 1999), suggesting that although the underlying mutations are not sufficient to drive malignancy, they are sufficient to allow some independence from the normal homeostatic mechanisms.

1.2.4.4.2 Role of nevi in melanoma development

Melanomagenesis is commonly described as the results of a de-differentiation process of transformed, mature melanocytes through nevus and dysplastic nevus stages to *in situ* and eventually invasive melanoma (Clark et al, 1984). According to the traditional model, during melanoma development, tumour cells escape keratinocyte control by various mechanisms that include down regulation of E-cadherin and upregulation of N-cadherin and MCAM. This shift in the expression of adherent proteins favours interactions of melanoma cells with endothelial cells and fibroblasts surrounding the tumour while disrupting the binding with keratinocytes (Haas et al, 2005). Upon specific activation, cells composing the malignant microenvironment promote these processes by secreting proliferative growth factors such as Insulin-like growth factor (IGF), Transforming Growth Factor beta (TGFβ), Basic Fibroblast Growth Factor 2 (bFGF) or Vascular Endothelial Growth Factor (VEGF) (Lee and Herlyn, 2007). Despite the five different stages of melanoma progression distinguishable histologically (benign nevi, dysplastic nevi, radial-growth phase (RGP), vertical growth phase (VGF) and metastatic melanoma (Figure 1.10), approximately three-quarters of melanomas develops in normal skin and less than one-half of the nevi associated with melanoma are dysplastic (Bevona et al., 2003). Thus, it is not clear whether it is more likely for a dysplastic nevus to develop in melanoma than any other type of nevus, and it is clear that a nevus precursor is not required for the majority of melanomas.

This phenomenon is difficult to explain, but in light of the CSC concept, an alternative hypothesis has been proposed. It can in fact be foreseen that melanocytes stem cells or immature progenitor cells present in the skin following the accumulation of malignant mutations will potentially give rise then to melanoma without progressing through intermediates. In addition, the tumour microenvironment composed of surrounding and/or recruited fibroblasts and endothelial and inflammatory cells contribute and support metastasis (Zabierowski and Herlyn, 2008) (Figure 1.16). The identification of molecularly defined melanoma SC should shed light on the cellular events driving melanomagenesis and may provide experimental systems for the discovery of novel melanoma therapies.

Figure 1.16 Schematic model of the potential relationship between melanocyte SC (MSC) and putative malignant melanoma SC (MMSC) in context of distinct physiological or malignant microenvironments. (**A**) The balance between Melanocyte SC (Pax3+, SOX10-, Mitf-) self renewal and differentiation, quiescence and proliferation in the hair follicle is regulated by Notch and/or Wnt signaling molecules. The homeostasis of these SCs is supposed to be maintained through the interaction with the physiological microenvironment, mainly composed of E-cadherin expressing keratinocytes). MMSX could arise by cumulative oncogenic mutation and/or loss of tumour suppressors. Surface marker associated with MMSC are ABCB5, CD133, CD20 and MMSC could secrete morphogens as Nodal or BMP4. Moreover, cleavages in the microenvironment may support MMSC formation and melanoma genesis. Malignant microenvironment is characterized by the production of proliferative growth factors such as bFGF, HIF, TGFβ, VEGF. Moreover, MMSC might alter the microenvironment directly trough secreted factors (**B**). (Adapted from Schatton and Frank, 2008).



1.2.5 Systemic therapy for melanoma

Surgical excision is the treatment of choice for melanomas, especially for early stages of disease, and systemic chemotherapy remains the most widely used treatment, especially when metastatic sites are not surgically accessible. Thus several studies investigating new drugs and exploration of mechanisms of tumour recognition by the patient's immune system are currently on-going with the final aim of offering to these patients new and more efficient therapeutic options.

1.2.5.1 Chemotherapy of melanoma

Traditional systemic chemotherapy has failed to significantly improve the survival of patients with non-resectable metastatic melanoma (Bajetta et al, 2002) but chemotherapy continues to have a role in palliation of disease. Although many agents have been tested, single-agent chemotherapy is generally considered ineffective (O'Day et al., 2002).

Dacarbazine (DTIC) is a pro-drug of the alkylating agent 5-(3-methyltriazen-1-yl)imidazole-4carboximide (MITC) that is generally well tolerated. It is the only cytotoxic agent that has been approved for the treatment of advanced melanoma and it is the standard against which new treatment have to be tested. DTIC and its response rate as single agent ranges from 15% to 25%, but these responses have in general brief duration (3-6 months) and the complete response rate is

only 5% (Lee et al, 1995). Because of this activity, DTIC has been tested alone or in combination therapy.

Temozolomide (TMZ) is a relative new alkylating agent, it is a pro-drug that spontaneously converts to MTIC under physiologic conditions (Stevens et al, 1987). Moreover, TMZ has extensive tissue diffusion including penetration of the blood-brain barrier and the cerebral spinal fluid (Newlands et al, 1992) and is commonly used as chemotherapeutic agent in the treatment of multiform glioblastoma (GBM) (Stupp et al, 2005). TMZ was shown to have a slightly higher response rate (13.5% vs 12.1%) and median survival (7.7 vs 6.4 moths) than DITC, neither of which was statistically significant (Middleton et al, 2000). TMZ cytotoxicity is mediate through methylation of DNA, and this adduct can be removed by methyl guanine methyltransferase (MGMT), a DNA repair protein that bypass the TMZ effects (Hegi et al, 2005). Studies combining TMZ with Thalidomide demonstrated a 32% response rate (Perussia et al, 1984), however the follow up study could not demonstrate a real improvement with this treatment (Clark et al, 2010).

The nitrosoureas are a group of alkylating agents that act by cross-liking DNA. Carmustine (BCNU) and lomustine have a response rate of 10-20%, similarly to DTCI, but are associated with more toxicity and their use is limited as single agents. Fotemustine is a nitrosourea that rapidly crosses the blood-brain barrier and it displays a 15% response rate that however was not associated woth survival advantage (Avril et al, 2004). The platinum compound (cisplatin and

carboplatin) have a modest activity against melanoma with a response rate of 15-20% (Gogas et al, 2004).

Microtubule toxins and microtubular disassembly have been demonstrated to have activity against metastatic melanoma. Vinblastine, a vinca alkaloid with modest activity and limited toxicity, has primarily been used in combination therapy. Vinorelbine did not demonstrate a significant effect alone (Jimeno et al, 2005; Whitehead et al, 2004) and had 20% response rate in a study in combination with tamoxifen (Feun et al, 2000). Paclitaxel and docetaxel are microtubule disassembly inhibitors with antitumour activity in a variety of neoplastic diseases. Paclitaxel has 12-16% response rate in previously untreated melanoma patients (Wiernik et al, 1993) and a 26% response rate when used in combination to carboplatin (Rao et al, 2006). Analogously, docetaxel alone showed a 12 % response rate (Bedikian et al, 2003) and its effect in combination with TMZ and cisplatin has also been recently described (Kim et al, 2009).

1.2.5.2 Target Agents in melanoma

The advent of new cellular and molecular techniques led to a precise knowledge of the signaling pathway abnormally activated in melanoma thus paving the ground for the development of target therapies. Target therapies exploit the activities of biological agents/drugs designed to selectively block a given cell signaling, to disrupt angiogenesis, growth and proliferation pathway and to enhance tumour apoptosis.

Vascular Endothelial Growth Factor (VEGF) has been implicated in tumour-induced angiogenesis, and its inhibition can suppress the tumour growth in murine models. Bevacizumab is a humanized murine monoclonal antibody that binds to VEGF shown to have activity in colon, lung, and breast cancer (Van Meter and Kim, 2010). Bevacizumab has been used in combination with several agents. Bevacizumab plus Paclitaxel showed an overall survival at 12 months of 43.3% suggesting some benefit from this treatment (Gonzalez Cao et al., 2007). Another study combined Bevacizumab with carboplatin and taxol, with a 17% of partial response rate and 57% of patients displaying stable disease lasting for 8 weeks (Perez et al., 2009).

Sorafenib is a tyrosine-kinase inhibitor with activity against the RAF serine/threonine kinas, although is also a multikinase inhibitor with activity against VEGFR-2 and-3. Sorafenib is FDA-approved for the treatment of renal cell carcinoma and hepatocellular carcinoma but in a melanoma study, no tumour

response was observed, although 19% of patient experienced stable disease (Flaherty et al. 2008).

A number of other small molecule inhibitors, including Raf265, XL281, AZ628, SB-590855 and PLX-4032 have been developed. These molecules are more selective than Sorafenib for RAF kinases, are much potent MAPK signaling inhibitors *in vivo* retaining good antitumour activity (Tsai et al., 2008). In particular, PLX-4720 resulted in being highly selective for BRAFV600E and preclinical experiments demonstrated that PLX4032 (RG7204) selectively blocked the RAF/MEK/ERK pathway in BRAFV600E mutant cells and caused regression of BRAF mutant xenografts. In a phase I-II trial PLX4032 induced complete or partial tumour regression in 81% of patients who had melanoma, with progression free survival of more than 7 months and manageable side effects (Flaherty et al., 2010). As expected, patients negative for the BRAFV600E mutation failed to respond to the treatment and no effect of PLX4032 was observed on nevi progression or regression. Although the number of patients in this trial was small, and eventually all patients experienced tumour relapse, nevertheless this study strongly suggests that selective targeting of early driver mutations can induce a substantial tumour regression in melanoma patients (Wellbrock and Hurlstone, 2010).

1.2.5.3 Immune-based therapy

Several observations suggested that the host generates an immunogenic response to melanoma, leading to the interest in exploring therapeutic modalities to enhance such a response

The most important therapeutic agent for melanoma treatment has been the T-cell growth factor IL-2. IL-2 has not a direct antitumour activity, and exerts its function through its ability to stimulate an immunological response by lymphocytes. Initial studies reported an overall response rate of 16% with 6% complete response rate (Lotze et al., 1986). Other studies reported overall response rate of 15-20% with durable responses (Atkins et al, 1999). In light of these findings IL-2 has been approved by the FDA as treatment for metastatic melanoma but the toxicities associated with high-dose IL-2 have been the main obstacle to its widespread use in the treatment of patients.

Interferons (IFN) are a complex family of proteins with immunomodulatory and antiangiogenic properties that are produced in response to viral infection or after T-cell activation. Their effects include upregulation of the Histocompatibility antigen expression on tumour cells and activation of various effector cells including natural killer (NK), T cells, monocytes and Dendritic Cells (DC). IFN was initially studied in 1980s and was associated to an overall response rate of 16%, with durable response generally achieved by patient with a small-volume cutaneous or soft tissue disease (Agarwala and Kirkwood, 1996). IFN-α has been used in combination with other agents, with higher response rates in the

IFN-α containing regimens (Hernberg et al., 1999; Hwu et al, 2006). Currently, IFN-α is used now in adjuvant therapy, in the treatment of patient after surgical resection of early stage melanoma, to prevent recurrences (Ascierto and Kirkwood, 2008).

New, innovative immune-based treatment strategies for melanoma include those that antagonize receptors that suppress the host immune response against the tumour (Cytotoxic T-lymphocyte antige-4 CTLA-4, Programmed Death-1, PD-1) and those that activate receptors that amplify the immune response (CD40 on antigen presenting cells, CD137 and OX40 on T cell). Human anti CTLA4 antibodies (ticilimumab, iplimumab) have been tested in human clinical trials. Animal studies first provided insights into the ability of an anti-CTLA4 antibody to cause tumour regression. Early clinical studies defined ipilimumab pharmacokinetics and possibilities for combination therapeutic regimen. Phase II trials of ipilimumab in advanced melanoma showed objective reponses and, in a phase III trial, ipilimumab was the first agent to demonstrate an improvement in overall survival (10.1 months) in patients with advanced melanoma also comparing ipilimumab action with gp100 peptide vaccine alone (6.4 months) or in combination treatment of ipilimumab plus vaccine (10.0 months). In 10-15% of the treated patient severe immune related adverse events occurred, but most this treatment-related toxicity was reversible with appropriate care (Hodi et al., 2010).

Vaccines for melanoma are designed to boost immune reactions against a malignancy that is already established; the vaccine with the highest response rate (7.1%) included DC or the addition of IL-2 to vaccines (Smith et al., 2004).

Best response rate occurred in vaccinated patients with subcutaneous or cutaneous disease. Most vaccines were successful in generating easily detectable, vaccine-specific immune response. Several small studies have explored the efficacy of tumour-associated antigenic peptides (gp100, tyrosinase) with or without IL-2, DC or other adjuvant, with no relevant clinical efficacy, even if peptide-specific T cells were induced in most patients.

CHAPTER 2: AIM OF THE STUDY

The cancer stem cells theory has changed our vision of how the human tumours are organized. Novel accumulating evidence support the idea that tumour cell heterogeneity is organized in a hierarchical mode and that a subpopulation of cells composing the tumour mass is responsible for the initiation and progression of tumour growth. The observation that the functional features of tumour initiating cells resemble those of stem/progenitor cells (proliferation capacity, self-renewal, and ability to sustain a differentiation program giving rise to a heterogeneous progeny) lead to the definition of tumour initiating cells as "Cancer Stem Cells" (CSCs). The isolation of cells required for tumour maintenance and progression is crucial to investigate new aspects of tumour biology. Furthermore, a molecular and phenotypic description of CSCs is essential for the identification of novel diagnostic and prognostic factors and to develop new therapeutic interventions targeting the CSC subpopulation within the tumour mass. One of the approaches to isolate CSCs exploits the ability of cells with stem cell properties to form non – adherent colonies when seeded at low density in a suitable culture medium.

Cutaneous melanoma is one of the most aggressive human tumours endowed with the capacity to metastasize very early in tumour pathogenesis and to disseminate to the draining lymph node/s and to the visceral organs by the haematogenous route. Melanomas are believed to arise from a mature differentiated melanocyte upon malignant transformation but in humans little

information is available about the development of normal melanocyte, even if the existence of melanocyte precursors in a localized region of the hair follicle known as the hair bulge has been described. Experimental evidence suggests that melanomas display a high degree of heterogeneity and plasticity *in vitro* and *in vivo*, retaining the ability to differentiate into a wide range of cell lineages and suggesting a possible origin from a cell with multilineage differentiation abilities.

Based on such considerations, it is now possible to foresee a CSC model also for human melanoma, and the present study is aimed at identifying and characterizing the putative CSCs in this human tumour. The following specific objectives have been addressed in this thesis:

- Isolation of melanoma cells able to grow as floating melanospheres in medium suitable for stem cell *in vitro* maintenance.
- Evaluation of the self-renewal capability of melanospheres, assessed as the ability to re-form spheres starting from one hundred or from a single cells.
- Assessment of the multipotency, seeding melanosphere-derived cells culture media specific for mesenchymal lineage differentiation.
- Evaluation of the melanosphere phenotype, with particular attention to the expression of melanoma and stem cell-associated markers. The expression of ABC drug transporter family was also studied considering the possible role of these proteins in melanoma drug resistance.
- Estimation of the tumour initiating capacity of putative melanoma stem cells by serial injection into immunocompromised mice of low dose melanosphere-derived cells with subsequent histological evaluation of xenografted tumours.

- Immunological properties of the isolated putative melanoma stem cells

To further highlight specific features distinguishing putative melanoma stem cells (melanospheres) from non stem-melanoma cells (adherent cells), the above listed analysis were performed on melanospheres and on the corresponding melanoma cells growing as an adherent monolayer in medium with the addition of FCS.

CHAPTER 3: MATERIALS AND METHODS

3.1 Melanoma cell cultures

3.1.1 Establishment of melanoma cell lines from tumour specimens

Lymph node melanoma metastases were collected upon patients' informed written consent according to the Internal Review and the Ethics Boards of the Istituto Nazionale Tumouri of Milan, Italy. Melanoma metastatic lesions were mechanically dissociated and live cells seeded in RPMI (Lonza) supplemented with 10% FCS (PBI), penicillin/streptomycin (Sigma), HEPES buffer (Lonza), and maintained in 5% CO_2 at 37° C at a the density of 1E6 live cells per 75 -cm^2 flask. Melanoma cells grew adherent to plastic forming confluent monolayers, and they were serially passed by trypsin disaggregation obtaining the melanoma cell lines named Me204ADH and Me14346ADH. The melanoma cell line MeJR8ADH was derived from a melanoma lung metastasis, and it has been kindly provided by Dr. G. Zuppi, Istituto Regina Elena, Rome. The fresh melanoma cell suspension derived from lymph node metastasis. Me15888 failed to establish an adherent melanoma cell line.

3.1.2 Selection of melanoma cells growing as non-adherent melanospheres

Melanospheres Me204S and JR8S were derived from their corresponding adherent melanoma cell line adapted to grow in 10% FCS by plating at clonal density (2500–5000 cells/cm^2) single melanoma cells grown as monolayer (Me204ADH and MeJR8ADH) into SCM consisting of Dulbecco's modified Eagle's medium/F-12 (Lonza) supplemented with 20 ng/ml epidermal growth factor (EGF, Peprotech) and 10ng/ml basic fibroblast growth factor (b-FGF; Peprotech), penicillin/streptomycin (Sigma). Melanospheres were maintained in 5% CO_2 at 37° C by adding fresh medium every 2-3 days. Primary melanospheres were then mechanically dissociated into single cells and plated at clonal density in SCM. To generate Me14346S and Me15888S melanospheres, fresh melanoma cells obtained after mechanical dissociation of post-surgery specimens were directly seeded at the same clonal density in SCM, maintained and dissociated as Me204S and MeJR8S melanospheres. Alternatively, melanoma cells were cultured in mesenchymal stem cell medium STEMPRO® MSC SFM medium (Gibco) or in the Neural stem cell medium Neurobasal™ (Gibco) supplemented with N-2 Supplement (Invitrogen) at the same density described above and following the same criteria for melanosphere dissociation.

3.2 In *vitro* assays

3.2.1 Cytofluorimetric analysis of melanoma phenotype

Cell phenotype was performed using the mouse anti-human primary antibodies reported in Table 3.1. For surface staining, 1-5E5 cells/sample were incubated with primary antibody for 30 minutes at 4℃ except for C D126 and CD130 that were incubated at Room Temperature (R.T.). After washing in culture medium, cells stained with purified antibody were then incubated with secondary antibody anti-mouse Alexa488 (Invitrogen).

To analyze intracellular antigens, cells were fixed with 100µl/sample of BD Cytofix/Cytoperm solution (Becton Dickinson, BD) for 20 minutes at 4℃. After fixing, cells were washed with Perm/Wash buffer 1x (BD) and then incubated with primary mouse anti-human antibody and secondary anti-mouse Alexa488 antibody as described above.

Nuclear antigens (Human Embryonic Stem Cell Marker Antibody Panel, R&D) were analyzed on cells fixed with 4% paraformaldehyde and permeabilized with TX-100 0.1% in phosphate-buffer saline (PBS) for 30 minutes at R.T. Staining was performed following the manufacturer's instructions and anti-goat or anti-mouse Alexa488 (Invitrogen) were used as the secondary antibody. Stained cells were analyzed by flow cytometry (FACScalibur, BD) and analyzed using FlowJo, V 8.5.2 (Treestar). Cells stained according to the protocol described above, were seeded on microscope slides by cytospin at 400rpm for 5 minutes and let them dry up for 4

hours. When spots were dry, cover slips were mounted with 20% Mowiol (Sigma) and cells were analyzed under fluorescent microscope (Nikon Elipse 600).

Table 3.1 List of Antibodies used for FACS analysis.

Anti-human Antibody	Cellular localization	Fluorocrome	Host species	Factory
CD9	Cell surface	Purified	Mouse	R&D
CD20	Cell surface	FITC	Mouse	BD
CD24	Cell surface	FITC	Mouse	BD
CD44	Cell surface	FITC	Mouse	BD
CD73	Cell surface	Purified	Mouse	BD
CD80	Cell surface	PE	Mouse	BD
CD86	Cell surface	PE	Mouse	BD
CD126 (IL-6Rα)	Cell surface	Purified	Mouse	BD
CD130 (gp130)	Cell surface	Purified	Mouse	BD
CD133/2	Cell surface	PE	Mouse	Miltenyi
CD146 (MCAM)	Cell surface	Purified	Mouse	BD
CD152 (CTLA-4)	Cell surface/ Cytoplasm	PE	Mouse	BD
CD166 (ALCAM)	Cell surface	Purified	Mouse	BD
CD181 (CXCR1)	Cell surface	APC	Mouse	BD
CD182 (CXCR2)	Cell surface	APC	Mouse	BD
CD192 (CCR-2)	Cell surface	APC	Mouse	BD
CD271 (NGFR)	Cell surface	Purified	Mouse	BD
CD274 (PD-L1)	Cell surface	PerCP	Mouse	BD
CD279 (PD-1)	Cell surface	FITC	Mouse	BD
IL-10Rα	Cell surface	PE	Mouse	R&D
SSEA-1	Cell surface	Purified	Mouse	R&D
SSEA-4	Cell surface	Purified	Mouse	R&D
Nestin	Cytoplasm	Purified	Mouse	R&D
Mart-1	Cytoplasm	Purified	Mouse (clone M-27)	Kawakami et al, 1997
Gp100 (HMB45)	Cytoplasm	Purified	Mouse	Dako

Anti-human Antibody	Cellular localization	Fluorocrome	Host species	Factory
Sox2	Nucleus	Purified	Mouse	R&D
Oct3/4	Nucleus	Purified	Goat	R&D
Nanog	Nucleus	Purified	Goat	R&D
Musashi-1	Nucleus	Purified	Mouse	R&D

3.2.2 Cytokines Bead Array (CBA)

Cell culture supernatants were collected after elimination of cells trough centrifugation and stored at -20℃. Cytokines and c hemokine concentration in culture supernatants were analyzed using the Human Th1/Th2 cytokines 11plex and the Human chemokines 6plex kits (Bender MedSystem) according to the manufacturer's instruction. Briefly, 25µl of cell culture supernatants were added to 25µl of assay buffer and to 25µl of the biotin-conjugated beads in polystyrene FACS tubes. Mixtures were incubated at R.T. for two hours. After washing, 50µl of streptavidin-PE solution was added and incubated for 1hour at R.T. After washing, samples were acquired using a FACSCalibur (BD). FACS raw data were analyzed with the specifically designed FlowCytomixPro Software (BenderMedSystem).

3.2.3 Sphere forming assay and clonogenic assay

For the Sphere Formation Assay (SFA), 100 single viable cells obtained from melanosphere dissociation were plated in 500µl of SCM in a 48-well plate coated overnight with poly-Lysine (Sigma). Cells were fed every 2-3 days by adding fresh SCM; after 5-8 days melanospheres were counted. For the clonogenic assay,

100µl of SCM containing 2.5 cells/ml were seeded in 96-multiwell plate. After 2-4 hours, each well was examined under the microscope and wells containing a single cell were then checked for sphere formation over the following days. In some experiments, viable melanoma cells were sorted according physical parameters thorough (FCSAria, BD) and seeded one cell/well into a 96 well plate.

3.2.4 Differentiation assay

Melanoma cells were induced to differentiate into adipocytes, chondrocytes and osteocytes according to the culture condition and staining procedures indicated in the Human Mesenchymal Stem Cell Functional Identification Kit (R&D).

For Melan-A staining 60.000 viable cells/cm^2 were plated in Lab-Tek chamber slides (Nunc) in RMPI+10%FCS for 2-4 days, until they were sub-confluent. Culture medium was removed and cells fixed with 4% PFA for 10 minutes at R.T. After washing with PBS containing 0.5% of Bovine Serum Albumin (PBS-BSA 0,5%), cells were incubated with a PBS-BSA 2% solution for 20 minutes at R.T. and subsequently incubated with 100µl/well primary anti-Melan-A/Mart-1 antibody for 60 minutes at R.T. After washing, cells were incubated for 60 minutes at R.T. in the dark with 300µl/well of a PBS-BSA 2% solution containing the anti-mouse Alexa488 secondary antibody diluted 1:300 (Invitrogen); nuclei were stained for 10 min in the dark with 300µl/well of a PBS-BSA 2% solution containing Hoechst33342 at final concentration of 5ug/ml (Sigma). After washing, cover slips were mounted with 20% Mowiol in H_2O (Sigma) and cells analyzed under a fluorescence microscope (Nikon Elipse 600).

3.2.5 Real Time PCR

3.2.5.1 *RNA extraction*

RNA extraction and digestion with DNase were carried out with the RNAqueous®-4PCR Kit (Ambion Europe LTD, Huntingdon, UK), according to the manufacturer's instructions. Obtained RNA was quantified by NanoDrop (Thermoscientific). RNA purity and integrity were assessed loading 500ng of extracted RNA on gel 1% agarose gel in TBE Buffer1x and running samples for about 30 minutes at 80V. Extracted RNA was then stored at -80℃.

3.2.5.2 *cDNA synthesis*

cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 1μg of RNA was mixed with the 2x master mix (10xRT buffer; 25x dNTP mix 100mM; 10x RT random primers; MultiScribe™ Reverse Transcriptase; RNase inhibitors; Nuclesae-free H_2O) into each sample. The reaction mixture was incubated at 25℃ for 10 minutes, followed by 120 minutes at 37℃ and then by heat inactivation of the enzyme at 85℃ for 5 sec in a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems).

3.2.5.3 *Real Time PCR*

The real-time PCR reaction was performed by taking advantage of TaqMan® Human ABC Transporter Arrays (Applied Biosystems), containing assays for 50 human ABC transporter family genes in addition to 14 endogenous controls in 384-well arrays. For the detection of immune-related genes the TaqMan® Human Immune Array (Applied Biosystems) was used.

Briefly, in every port of the array cDNAs (2µl), TaqMan Universal PCR Master Mix (Applied Biosystems, 50µl) and nuclease-free water (48µl) were loaded. The reactions were performed using the ABI 7900HT instrument (Applied Biosystems).

The relative levels of templates in each sample were determined through Relative Quantitation (RQ) using comparative CT (ΔΔCT) assay configuration ($RQ=2^{-\Delta\Delta^{CT}}$, where $\Delta CT=CT_{target\ genes} - CT_{GAPDH}$, and $\Delta\Delta CT=\Delta CT_{sample} - \Delta CT_{calibrator}$).

The *GAPDH* gene was used as endogenous control. Data analysis was performed with SDS (Sequence Detection Systems) 2.2.2 software (Applied Biosystems).

3.2.6 Molecular characterization of melanospheres and adherent cells

3.2.6.1 Cellular extraction DNA

The cells (2E6 – 4E6) were centrifuged at 1800 rpm for 5 minutes. The obtained pellet was washed with PBS and DNA extracted with the kit Qiamp DNA Blood Mini Kit (Quiagen) following the protocol for purification of DNA from Blood and Body Fluid. DNA was been eluted in 200µl of Buffer AE (in the Kit) and stored at 4° C. The quantity and quality of DNA were measured by assessment of absorbance at 260 and 280 nm (Nanodrop, Labtech International).

3.2.6.2 Homozygous Deletion Analysis

To assay for homozygous deletions of the *CDKN2A* gene, a comparative multiplex polymerase chain reaction technique was used. Globin was amplified as a control gene, and three exons of *CDKN2a* gene and the first exon of p14 gene were amplified. Primers, temperature annealing (TA) and length of fragments obtained by PCR are reported in Table 3.2. We used 50ng of genomic DNA in final volume of 20µl Mix containing 1X of Buffer 10X, 0.1mM dNTP 2mM, 1,5mM di $MgCl_2$ 25 Mm, 0.2pM oligo1 10pM, 0.4pM oligo2 10pM and 1unit Ampli Taq 5 U/µl. PCR reactions were set with an initial denaturation of 5 minutes at 95℃ and subsequent denaturation for 30s at 95℃, annealing for 30 seconds at 57℃ and extension for 30 seconds at 72℃. Thirty-five cycle s were used to amplify the PCR products that were separated by electrophoresis on 3% agarose gels and visualized under ultraviolet light by ethidium bromide staining.

Table 3.2 List of primer pairs and PCR conditions for homozygous deletions analysis.

AMPLICON NAME	OLIGO NAME	SEQUENCE	EXON	TA	LENGTH
GLOBINA	GLO FW GLO RW	5'-CTT CTG ACA CAA CTG TGT TCA CT- 3' 5'-TCA CCA CCA ACT TCA TCC ACG T-3'	1	57°	120bp
p14	1B FW IB RV	5'-TCC CAG TCT GCA GTT AAG -3' 5'-GTC TAA GTC GTT GTA ACC CG -3'	1β	57°	488bp
p16/CDKN2A	P1611F P1613R	5'-CAG AGG ATT TGA GGG ACA GG-3' 5'-AGA ATC GAA GCG CTA CCT GA-3'	1α	57°	403bp
p16/CDKN2A	P1622F P1625R	5'-AGC TTC CTT TCC GTC ATG C-3' 5'-GGA AGC TCT CAG GGT ACA AAT TC-3'	2	57°	410bp
p16/CDKN2A	P1632F P1632R	5'-GCA GTG GAC TAG CTG CTG CTG GA-3' 5'-TTA CGG TAG TGG GGG AAG G-3'	3	57°	388bp

3.2.6.3 BRAF Mutational Analysis

Mutant allele-specific PCR was used to detect the *BRAF*^{V600E} mutation as previously described (Pollock et al., 2003). Briefly, two different forward primers with a single base substituted at the end of the primer, were used to amplify the wild-type allele and the *BRAF* mutation, respectively. The reverse primer used for both reactions was common. Temperature annealing (TA) and length of fragments obtained by PCR are reported in Table 3.3. Both PCR reactions were done separately in a 25µL reaction volume containing 1X of Buffer 10X, 1.5mmol/L $MgCl_2$, 0.1mM dNTP 2mM, 1,5mM di $MgCl_2$ 25mM, 50ng of genomic DNA, 0.2pM oligo 10pM, and 0.25 units of Ampli Taq 5 U/µl . Both PCR reactions were set with an initial denaturation of 7 minutes at 95℃ and su bsequent denaturation for 30 seconds at 95℃, annealing for 30 seconds at 60℃, and extension for 30 seconds

at 72℃. Forty cycles were used to amplify the PCR products that were separated by electrophoresis on 3% agarose gels and visualized under ultraviolet light by ethidium bromide staining. Two controls, one mutated and one wt were included in PCR amplifications.

Table 3.3 List of primer pairs and PCR conditions for BRAF mutational analysis.

AMPLICON NAME	OLIGO NAME	SEQUENCE	EXON	TA	LENGTH
Braf wt	**Braf wt** fw	5'-ACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGT- 3'	15	60°	198 bp
	Braf wt rw	5'-CTATGAAAATACTATAGTTGAGACCTTCAATGACTT-3'			
Braf mut	**Braf mut** fw	5'- ACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGA -3'	15	60°	198 bp
	Braf wt rw	5'-CTATGAAAATACTATAGTTGAGACCTTCAATGACTT-3'			

3.2.7 Western Blot analysis

3.2.7.1 Protein extraction and quantification

Melanospheres were collected and dissociated as indicated in section 3.2.1 and 4E6 cells were centrifuged to obtain a pellet. Cell pellets were washed twice in cold Phospahte Buffer Saline (PBS) and lysed for 30 minutes on ice in Ripa-Like buffer (50nM Tris, 250mM NaCl, 0.1% SDS, 0.5% NP40, 2mM DTT) containing the protease inhibitor Pepstatin 5mg/ml (USB), Leupeptin 10mg/ml, Aprotinin 1mg/ml and PMSF 10mM (Sigma). After lysing, samples were centrifuged at 12000 rpm for 15 minutes at 4℃ to recover the protein fraction in the supernatant. Protein quantification was calculated using the BCA colorimetric assay (Bio-Rad). Briefly, equal volume of samples and serial dilutions of a solution containing a known

concentration of Albumin from Bovine Serum (BSA) were seeded in a 96 well plate and subsequently copper sulphate and bicinchoninic acid (BCA) were added to the samples. After 15 minutes of incubation at R.T. absorbance at 550nm of wave length was read at the spectrophotometer; a calibration line obtained measuring absorbance with known concentration of the BSA sample, was used to calculate the protein concentration of each sample.

3.2.7.2 Electrophoresis

For Western Blotting, 60µg of proteins were denaturated for 10 minutes in a solution containing 0.5M DTT and LDS sample buffer (SDS, bromophenol blue, glycerol, Invitrogen) and separated on 4-12% Bis-Tris precasted gels (Invitrogen) in Running Buffer (MOPS-SDS 20x: MOPS 1M, Basic Tris 1M, SDS 69.3mM, EDTA 20.5mM) using a vertical electrophoresis apparatus (Novex mini cell; Invitrogen) at 120V for about 2 hours.

3.2.7.3 Protein Transfer and signal detection

After electrophoresis, protein were transferred to Nitrocellulose membrane (Hybond, Amersham) and embedded with Transfer Buffer (Bicin 500mM, Bis-Tris, 500mM, EDTA 20.5mM) using a semidry apparatus (Hoefer semiphor, Amersham) at 45mA for about 1h. Membranes previously saturated with PBS-milk 5% solution, were probed with human primary antibody (see Table 3.4) diluted in PBS-milk 0.5% O.N at 4℃. After washing in PBS Tween 0.1% milk 0.5%, the signal was detected using peroxidase-conjugated anti mouse antibody (BD) or anti-rabbit

secondary antibodies (Amersham) for 1h at R.T., washed and developed using the ECL Plus Western Blotting Detection System (Amersham). Photographic plates were developed using a developing machine (Curix 60, AGFA). As loading control, all membranes were also hybridized with a anti-vinculin antibody.

Table 3.4 List of primary antibodies used in WB analysis.

Anti-human Antibody	Host species	Concentration	Factory
PTEN	mouse	1:500	A.G. Scientific
AKT	mouse	1:500	Cell Signaling
pAKT (serin^{473})	mouse	1:500	BD
ERK1,2	rabbit	1:10000	Sigma
p-ERK1,2 (Thr^{183} and Tyr^{185} in ERK-2)	mouse	1:10000	Sigma
Vinculin	mouse	1:10000	Sigma

3.3 In *vivo* assays

3.3.1 Evaluation of tumorigenic potential

Seven week old SCID mice (Charles River) were intradermally injected with either 10^4, 10^3 or 10^2 melanosphere-derived cells or melanoma adherent cells resuspended in PBS and mixed with Matrigel (BD) at a 1:1 ratio. Viability of the injected cells was determined using the Trypan Blue exclusion test. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines. Mice were followed for up to 200 days or sacrificed when tumour mass was >1.5g. To perform serial transplantations, mice bearing tumours of about 500mg were sacrificed, their

tumour mechanically dissociated as reported for human tumour specimens and viable single cells were re-injected into SCID mice with the same modality as the first injection.

3.4 Ex-*vivo* assays

3.4.1 Immunoistochemistry

25 human tissue samples consisting of 11 primary melanomas, 14 metastatic melanomas and 70 xenograft tumours were analyzed by indirect immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (1-2μm) were incubated in methanol plus 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity, rinsed with water several times, and incubated with one of the monoclonal mouse anti-human primary antibodies listed in Table 3.5.

Antigen retrieval was performed in 0.05 mol/L sodium citrate (pH 6) for 6 min at 95℃ in an autoclave for Melan-A, HMB45 and Nestin; 0.05 mol/L sodium citrate (pH 6) for 15 min at 95℃ in an autoclave for NGFR; 0.05 mol/L sodium citrate (pH 6) for 30 min at 95℃ in an autoclave for CD133 and MITF; EDTA 6 min for CD166, followed by cooling to room temperature and final rinses in PBS. Nonspecific binding sites were blocked by incubation with Ultra V Block (Lab Vision) for 10 minutes at R.T. A secondary biotinilated anti-mouse antibody was incubated for 30 minutes at R.T. diluted 1:100 (Dako Cytomation). After washing, tissues were incubated 30 minutes at R.T. with peroxidase-conjugated streptavidin diluted 1:300 (Horseradish Peroxidase, Dako Cytomation). The peroxidase enzyme reaction was

developed with 3,3'-diaminobenzidine (Dako Cytomation). For negative controls, primary antibody was omitted.

Table 3.5 List of primary antibodies used in immunohistochemistry analysis.

Anti-human Antibody	Host species	Cellular localization	Dilution	Factory
Anti-Human Melanosome (gp100) clone HMB45	mouse	Cytoplasm	1:50	Dako
Melan-A (Mart-1) Clone A103	mouse	Cytoplasm	1:50	Dako
MITF Clone 34CA5	mouse	Nucleus	1:200	Novocastra Laboratories
Nestin	mouse	Cytoplasm	1:200	Chemicon
CD133/1 clone AC133	mouse	Cell membrane	1:25	Miltenyi
CD166 (MCAM) clone MOG/07	mouse	Cell membrane	1:10	Novocastra Laboratories
CD271 (NGFR) clone 7F10	mouse	Cell membrane	1:50	Novocastra Laboratories

3.4.2 Masson-Fontana staining for melanin detection

6μm thick deparaffinised and hydrated sections were cut and stained with Silver Nitrate solution for 1 hour at 56℃. When sections were light brown, they were rinsed with distilled water and then stained with gold chloride solution 0.2% for 10 minutes at R.T. After washing, sections were incubated with sodium thiosulfate solution 5% for 5 minutes at R.T. Washed sections are then dehydrated with xylol and observed under a light microscope (Nikon).

3.4.3 Transmission Electron Microscopy (TEM) analysis

11 tumours were fixed with 4% paraformaldehyde and 2% glutaraldehyde in phosphate buffer 0.12M. The samples were post-fixed with 1% OsO4 in cacodylate buffer, dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections (60nm), obtained with ultra microtome Ultracut E Reichert-Jung, were doubly stained with uranyl acetate and lead citrate and examined with a transmission electron microscope CM 10 Philips.

3.4.4 Statistical analysis

The differences in tumourigenicity (tumour take and latency) and in the tumour mass of the xenografts generated by melanospheres and adherent cells were statistically evaluated. For all the analyses, multiple regression models were used: a log-normal model for "time-to-event" data to evaluate differences in the tumorigenicity, and a generalized linear model for normal data in the case of tumour mass. The statistical assumptions were verified, and the log-normal model was chosen as being the best fit among a number of parametric alternatives (exponential, Weibull, and log-logistic models). The model predictors were treatment (melanospheres and adherent melanoma cells), cell line (Me204, MeJR8, and Me14346), and dose (10^4, 10^3, and 10^2). When treatment was the factor of experimental interest, cell line and dose represented study design related covariates. All these factors were categorical, and thus coded within the models by indicator (0–1) dummy variables. A search for interactions between treatment and

cell line or dose was also performed. Analyses were performed with SAS (Cary, NC); two-sided P-values of 00.05 were considered statistically significant.

CHAPTER 4: RESULTS

4.1 Melanoma cells form anchorage-independent, self-renewing and multipotent spheres in stem cell medium.

Normal and cancer stem cells can be selected *in vitro* by exploiting their ability to organize into anchorage-independent, three-dimensional spherical structures when cultured in serum-free medium supplemented with specific growth factors. In the present thesis, this same strategy has been applied with the aim of testing the hypothesis that cells with stem cell properties are indeed present in human metastatic melanoma and that these cells can be isolated *in vitro* either from melanoma cell lines already adapted to grow as an adherent monolayer or directly from fresh cellular suspension obtained by tissue processing of post surgery specimens.

4.1.1 Selection of cells growing in Stem cell medium (SCM)

For the purpose outlined above, 18 lymph node melanoma metastases were processed as described in materials and methods and viable melanoma cells obtained after mechanical and enzymatic digestion were seeded in SCM. SCM supported the selection of cells growing as melanospheres in 8 of these lesions with a success rate of 44%. A similar success rate of melanosphere formation (32%) was achieved when melanoma cell lines, previously established as adherent cells in FCS, were used as the source of melanoma cells to be seeded in SCM. Melanospheres were also obtained using commercial media for neural stem cells, while media for mesenchymal stem cells was less efficient in supporting melanosphere generation and growth (Table 4.1).

Table 4.1. Selection of melanospheres using different culture media.

Cell type	Origin of cells	SCM	Mesenchymal SC medium	Neural SC medium
Me204S	Adherent cells	+	+	+
MeJR8S	Adherent cells	+	+	+
Me14346S	Fresh suspension	+	+	+
Me15888S	Fresh suspension	+	+	+
Me104B	Adherent cells	+	+	+
Me304	Adherent cells	No spheres	No spheres	Short term spheres
Me8146	Adherent cells	Short term spheres	No spheres	Short term spheres
Me1	Adherent cells	No spheres	No spheres	No spheres
Me8	Adherent cells	No spheres	No spheres	Short term spheres
Me5127	Fresh suspension	Short term spheres	No spheres	Short term spheres
Me7902	Fresh suspension	Short term spheres	No spheres	Short term spheres
Me5988	Fresh suspension	Short term spheres	No spheres	Short term spheres
Me2179	Fresh suspension	Short term spheres	No spheres	Short term spheres
Me1390	Fresh suspension	No spheres	No spheres	No spheres

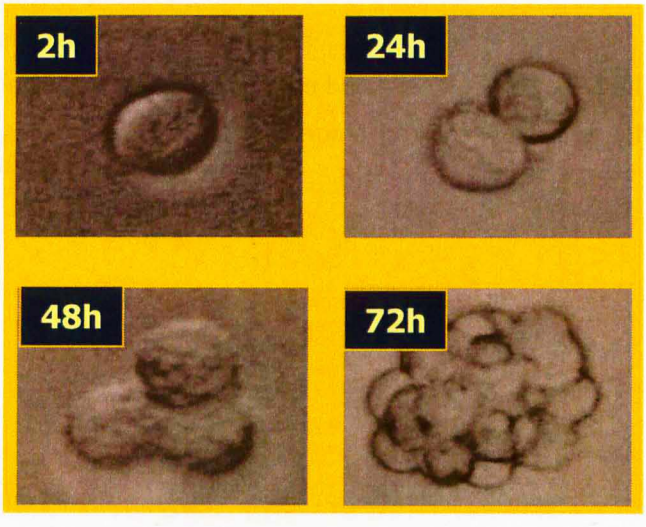
Four of these melanosphere cultures, two derived from adherent melanoma cell lines, namely melanospheres MeJR8S and Me204S, and two derived directly from lymph node melanoma metastasis, namely Me15888S and Me14346S, were kept *in vitro* by sequentially dissociating and re-seeding at low density in SCM medium for several passages (> than 30) and used in this thesis.

Viable melanoma cells obtained after mechanical and enzymatic digestion of metastatic lesion Me14346 were also seeded in medium supplemented with 10% of FCS and thus the corresponding adherent cell line, named Me14346ADH was also established. The fresh melanoma cell suspension derived from melanoma Me15888 failed to establish an adherent melanoma cell line and thus the adherent counterpart of Me15888S was not available for analysis.

4.1.2 Self-renewing capacity

Stem cells usually display an undifferentiated status and have the crucial features of self-renewal and multilineage differentiation. The self–renewing capacity of tumour spheres was evaluated by sphere formation and clonogenic assays. Both tests gave consistent results showing that all the melanospheres displayed a quite high frequency of self-renewing cells when seeded in SCM both at clonal density (SFA) or as single cells (clonogenic assay) (Figure 4.1).

Figure 4.1 Clonogenic assay. One single cell seeded in a well of a 96well-plate and, after subsequent cell divisions, gives rise to a melanosphere. Numbers indicate time (h=hours) in culture in SCM after single cell seeding.



To test whether cells endowed with self –renewal were also detectable inside melanoma cell lines growing as a monolayer, SFA and clonogenic assays were also performed with melanoma cells obtained by melanoma adherent cell lines after trypsin digestion. As reported in Table 4.2, self-renewing cells were also present in adherent melanoma cell lines but their frequency was significantly lower

than in the corresponding melanosphere-derived counterpart. These data suggest a strong enrichment of the self-renewing cells in melanospheres (Table 4.2).

Table 4.2. Self-renewal of melanospheres (S) and melanoma adherent cells (ADH).

Cell Type		SFA[1]	Clonogenic assay[2]
Me204	*S*	54 ±7.15[3]	52.7 ± 5.6
	ADH	10.7 ± 8.2	4.4 ± 0.3
MeJR8	*S*	25.6 ± 1.1	18.5 ± 2.1
	ADH	8.2 ± 3.8	6.4 ± 0.2
Me14346	*S*	26.9 ± 4.1	11.8 ± 4.2
	ADH	0.9 ± 1.1	0
Me15888[4]	*S*	14 ± 8.2	5.8 ± 1.8

[1] Percentage of melanospheres per 100 cells

[2] Percentage of clones per total single cells seeded

[3] Mean ± SD (Standard Deviation) calculated on the basis of three independent experiments

[4] Me15888 failed to establish an adherent melanoma cell line, thus the adherent counterpart of Me15888S was not available for analysis.

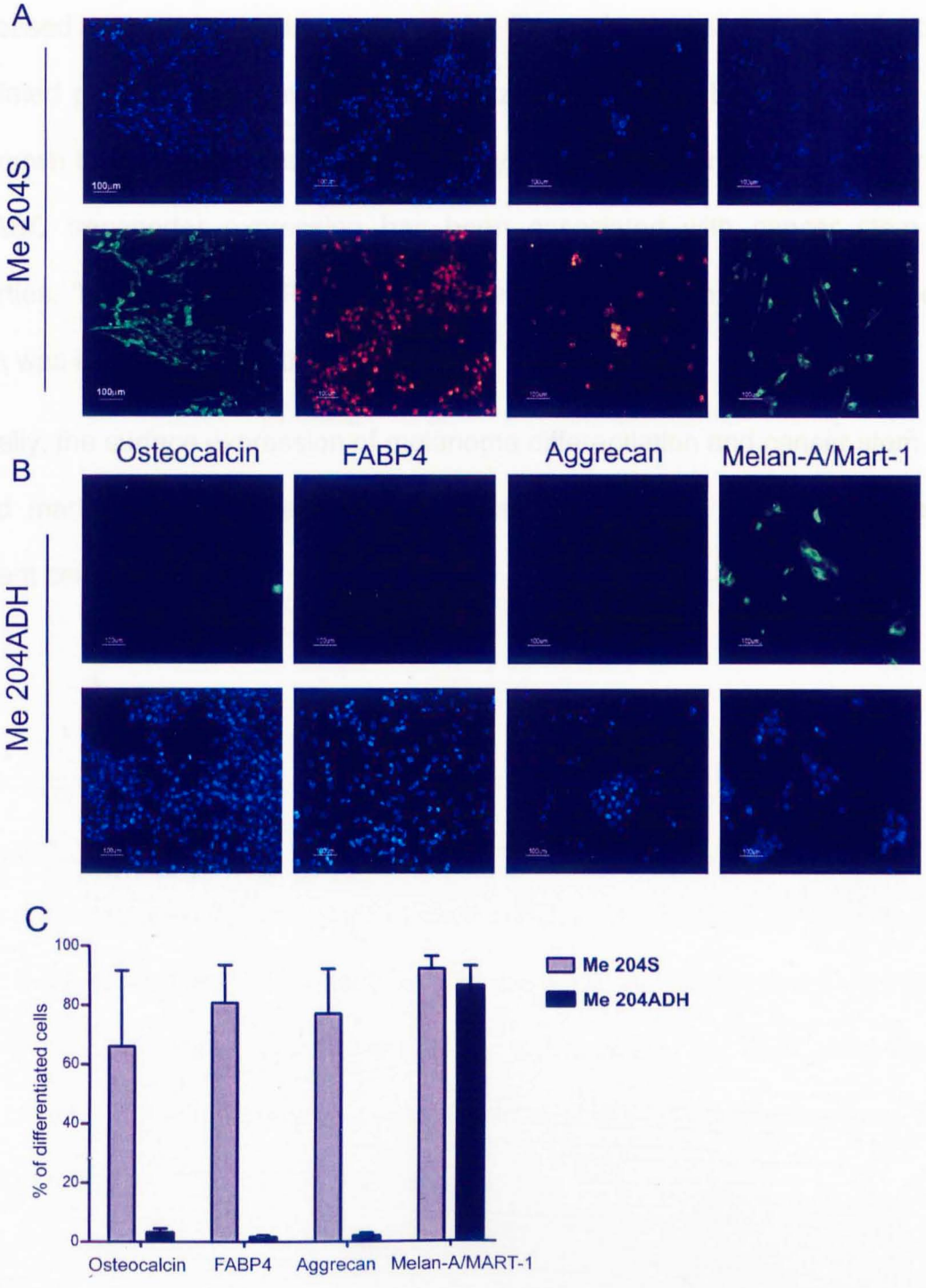
4.1.3 Multilineage differentiation capacity

Melanocytes derived from the pluripotent cells of the neural crest, that give rise also to cells of the mesenchymal lineage. In view of the fact that multipotency is one of the main features defining stem cells, in this thesis melanosphere-derived cells or their adherent counterpart were tested for the ability to differentiate into cells of mesenchymal origin by withdrawal of growth factors and culture in differentiating media specific for the cells of mesenchymal lineage. Differentiation was monitored by detecting the acquired expression of markers specific for each lineage, namely Osteocalcin for osteocytes, Fatty Acid Binding Protein-4 (FABP4) for adipocytes and Aggrecan for chondrocyte differentiation.

Canonical melanoma differentiation was assessed seeding melanosphere-derived cells into the medium for adherent melanoma cell lines, namely RPMI supplemented with 10% FCS and testing the expression of the Melan-A/MART-1, a marker for melanocytic lineage differentiation.

As shown in Figure 4.2, Me204S was able to differentiate in all the 3 cell types of mesenchymal origin with similar efficiencies, and it also expresses Melan-A/MART-1 upon seeding in 10%FCS. Conversely, the adherent counterpart Me204ADH did not display differentiation capacity for any of the mesenchymal lineages tested, while keeping a high expression of MelanA/Mart-1, supporting the notion that melanospheres are enriched in self–renewing multipotent cells.

Figure 4.2 Mesenchymal differentiation of melanospheres and melanoma adherent cells. (Me204S (A) and Me204ADH (B) cells were induced to differentiate in osteogenic, adipogenic chondrogenic media. Differentiated cells were then stained for Osteocalcin (green, Alexa 488), FABP4 (red, TRITC) or Aggrecan (red, TRITC), and with Hoechst33342 (upper and lower row in A and B). Melan-A/MART-1 staining was also performed after culturing Me204S and Me204ADH in medium with serum. (C) Histogram shows the percentage of Me204S (grey columns) and Me204ADH (black column) cells expressing the indicated differentiation marker counted in ten 20x random fields. Data reported mean ± SE (Standard Error) of three independent experiments were performed and each experiment was performed in triplicate. Scale bar = 100 µm.



4.2 Phenotypic characterization of melanospheres

To better characterize the growing melanospheres, the presence of the most common mutations associated to melanoma was checked by taking advantage of PCR-based techniques and, subsequently, the activation status of melanoma-associated proliferative pathways was analyzed by western blot. In addition, it is well known that melanoma is a tumour highly resistant to standard chemotherapy, and ABC transporter expression has been associated with cancer stem cell properties. Thus real time PCR analysis for the expression of ABC transporter mRNA was also included in this thesis.

Finally, the surface expression of melanoma differentiation and cancer stem cell-related markers was evaluated on melanospheres and on their corresponding adherent cell lines as well.

4.2.1 Mutational analysis and activation status of melanoma proliferative pathways on melanospheres and adherent cells

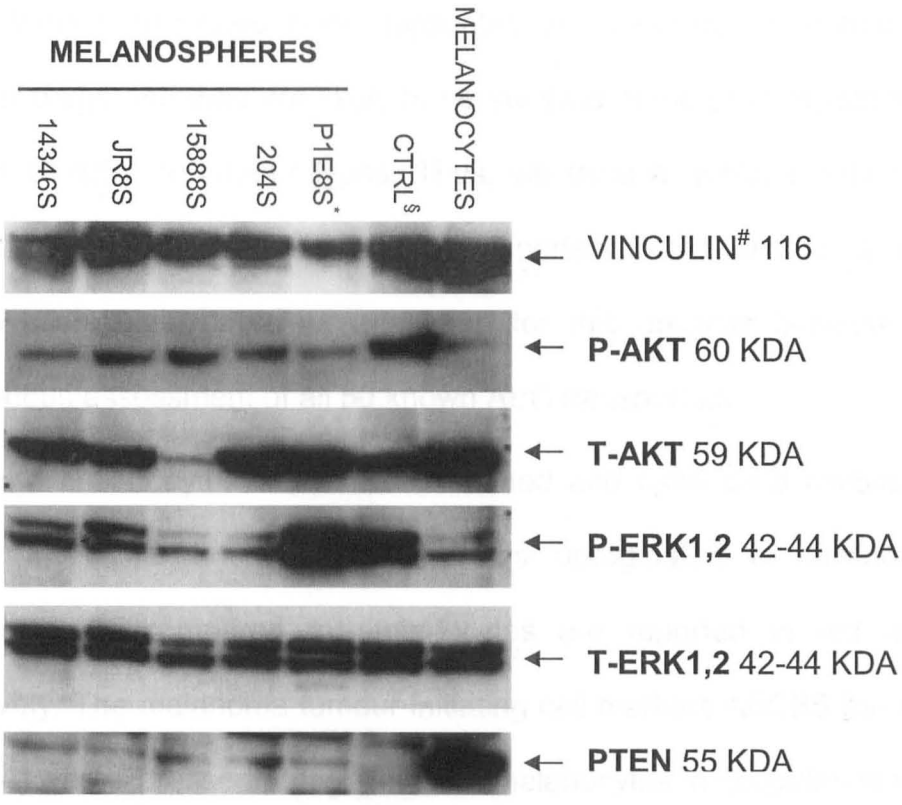
As reported in Table 4.3, the mutational status of melanospheres was variable and as absence of mutation in BRAF was associated with CDKN2A deletion.

Table 4.3. Melanoma common genetic alteration in melanospheres and adherent cells lines used in this study. S= melanospheres; ADH=Adherent cells; WT= Wild Type; MUT= mutated; POS= positive; HD=homozygous deletion.

Cell Type		BRAF ^{V600E}	CDKN2A
Me204	*S*	**wt**	**HD**
	ADH	**wt**	**HD**
MeJR8	*S*	**MUT**	pos
	ADH	**MUT**	pos
Me14346	*S*	**wt**	**HD**
	ADH	**wt**	**HD**
Me15888	*S*	**MUT**	pos

Conversely, as evidenced by western blot all the melanospheres lacked PTEN expression, consequently showing an enhanced level of activated AKT (p-AKT) as compared to melanocytes that instead were enriched in PTEN. PTEN deficiency is known to be associated with constitutive activation of AKT, which lead to enhanced proliferation and a more aggressive behaviour. Moreover, the ERK pathway was also activated in melanospheres as confirmed by the positive staining for p-ERK, the activated form of ERK (Figure 4.3).

Figure 4.3 WB analysis of signalling pathway activated in melanospheres. All the analyzed melanospheres express total (t-ERK) and activated ERK (p-ERK). Moreover, melanospheres express AKT (t-AKT) and its active form (p-AKT) while lacking PTEN that is widely expressed on melanocytes.



*:P1E8S is a MeJR8S single-cell derived clone. §: melanoma adherent cell line used as control. #: loading control.

4.2.2 Expression of ABC transporters on melanospheres and adherent cells

ABC transporters have been implicated in conferring resistance to multiple antitumor drugs and they are likely to be involved in the drug-resistant phenotype of CSCs of different tumour types. Thus, we used a genome-wide approach to examine the expression level of such transporters in our melanoma models. The TaqMan microfluidic cards were chosen for this analysis because they allow simultaneous assessment of all 50 known ABC transporters.

Cultured melanocytes were also examined and used as a calibrator for data analysis In Table 4.4. ABC transporters upregulated or downregulated in melanoma cells compared to melanocytes are reported in red and in blue respectively. The melanoma tumour initiating cell markers ABCB5 (green boxed in Table 4.4) was expressed at high level on melanocytes in accordance to published data (Heimerl et al., 2007). Me14346S melanospheres displayed an enhanced level of ABCB5 with respect to their adherent counterpart (Me14346ADH) (Figure 4.4). Different components of the ABCC subfamily –recognized as crucial for the multidrug-resistant phenotype (Gatti et al., 2009) - displayed enhanced levels in the three melanosphere models as compared to the corresponding adherent cells (ABCC1, ABCC2, ABCC4, pink-boxed in Table 4.4), whereas ABCG2 (yellow boxed in Table 4.4), a well known marker for CSC of different tumour types, was not up-regulated.

Table 4.4. Detection of ABC transporter mRNA in melanospheres and adherent cells. Melanocytes were used as calibrator.

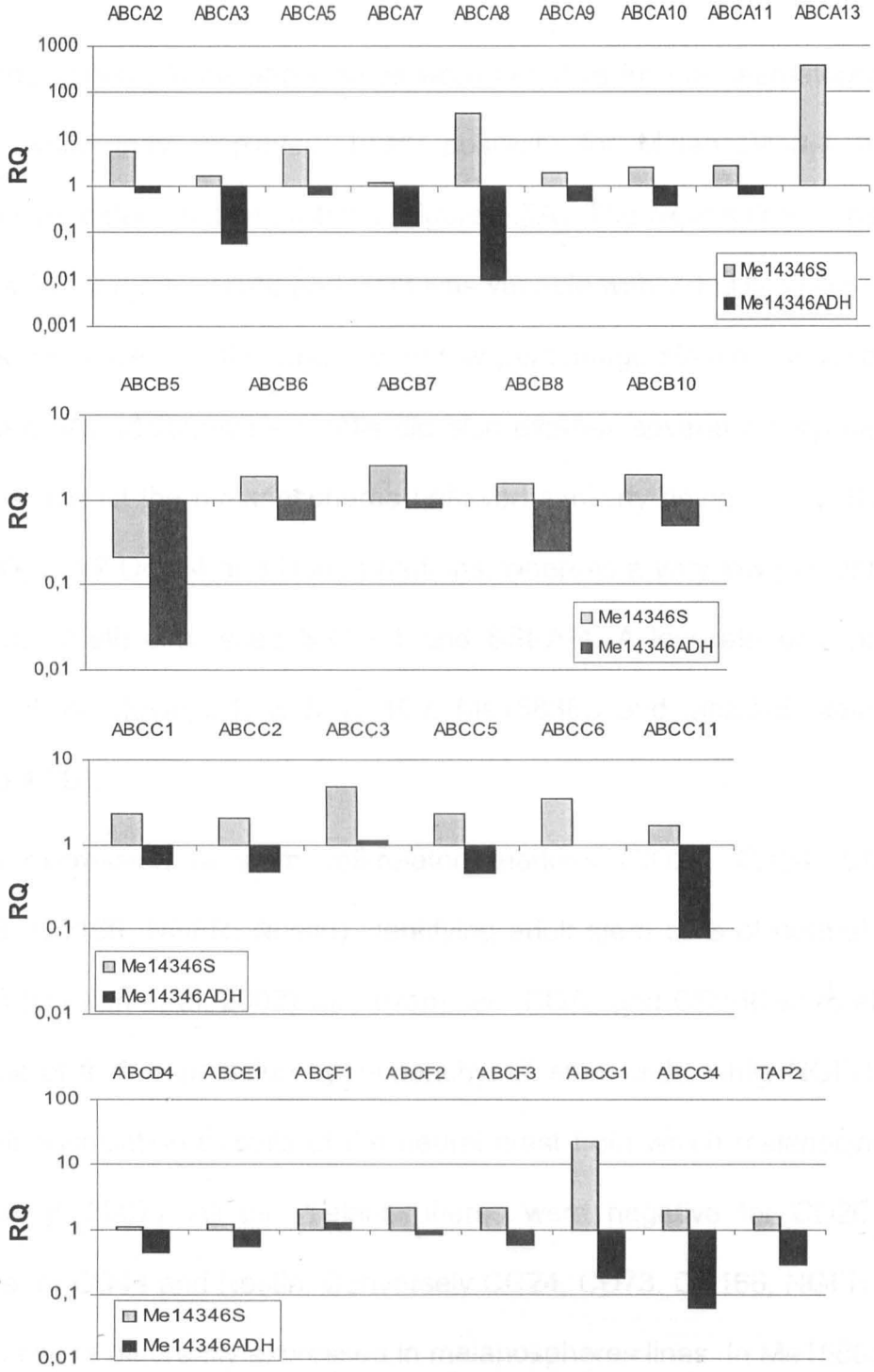
	Me14346		Me204		MeJR8		Me15888	[1]Melanocytes
	ADH	*S*	*ADH*	*S*	*ADH*	*S*	*S*	*ADH*
	[2]RQ	RQ	RQ	RQ	RQ	RQ	RQ	RQ
ABCA1	0,12	0,19	0,05	0,36	2,39	0,09	0,05	1
ABCA2	0,74	5,7	3,79	8,94	4,02	3,02	6,65	1
ABCA3	0,06	1,68	33,71	6,94	12,17	0,62	243,97	1
ABCA4	3081,38	[3]-	683,95	-	-	-	1860,63	1
ABCA5	0,64	5,74	0,5	5,01	0,89	1,57	3,35	1
ABCA6	0,07	0,16	-	0,19	0,16	0,06	0,61	1
ABCA7	0,14	1,21	0,72	0,63	0,56	0,25	5,73	1
ABCA8	0,01	36,89	-	0	0,06	0,02	-	1
ABCA9	0,49	1,93	-	0,74	-	0,27	0,68	1
ABCA10	0,37	2,36	-	3,31	0,63	0,7	3,7	1
ABCA11	0,64	2,67	0,57	0,49	0,44	0,22	0,71	1
ABCA12	0,63	0,58	113,96	-	-	-	-	1
ABCA13	-	392,35	0	1,12	-	-	-	1
ABCB1	1,55	0,1	0,3	0,66	0,03	0	107,35	1
ABCB4	0,48	0,13	0,01	0,01	0,16	0,01	1,3	1
ABCB5	0,02	0,21	0	0	0	0	0	1
ABCB6	0,59	1,91	0,61	1,36	0,6	0,63	1,12	1
ABCB7	0,82	2,53	0,63	1,9	0,87	0,59	2,63	1
ABCB8	0,25	1,54	0,27	0,57	0,45	0,39	1,13	1
ABCB9	0,33	0,58	0,56	0,1	0,57	0,69	1,29	1
ABCB10	0,49	1,93	0,36	0,02	0,73	0,86	4,4	1
ABCB11	-	-	-	-	-	-	-	-
ABCC1	0,58	2,32	0,54	1,4	1,01	0,82	1,52	1
ABCC2	0,48	2,01	0,02	1,05	0,45	0,6	2,77	1
ABCC3	1,11	4,79	323,27	36,56	0	0	0	1
ABCC4	1,17	1,47	0,17	2,03	1,22	1,06	3,09	1
ABCC5	0,46	2,35	0,84	1,47	0,97	0,62	4,03	1
ABCC6	-	3,43	4,25	0,71	1,5	0	3,06	1
ABCC8	-	1940,61	6601,22	-	-	-	0	-
ABCC9	-	-	-	-	-	-	2,97	1
ABCC10	0,23	0,98	0,14	0,83	0,44	0,36	2,16	1
ABCC11	0,08	1,68	0,64	-	-	0,14	2,68	1
ABCC12	-	-	-	-	-	-	-	1
ABCC13	-	-	-	-	-	-	-	1
ABCD1	0,4	0,38	0,2	1,6	1,23	0,96	4,6	1
ABCD2	1,02	0,82	-	-	1,16	0,35	-	1
ABCD3	1,02	1,25	0,25	0,81	0,32	0,41	0,43	1
ABCD4	0,43	1,06	0,77	1,14	0,73	0,42	3,57	1

	Me14346		Me204		MeJR8		Me15888	[1]Melanocytes
	ADH	*S*	*ADH*	*S*	*ADH*	*S*	*S*	*ADH*
	[2]RQ	RQ	RQ	RQ	RQ	RQ	RQ	RQ
ABCE1	0,55	1,23	0,44	0,89	0,45	0,48	1,29	1
ABCF1	1,28	2,05	0,71	1,21	0,7	0,67	2,16	1
ABCF2	0,83	2,18	2,1	1,21	0,77	0,75	0,95	1
ABCF3	0,57	2,18	0,78	0,5	0,5	0,41	1,67	1
ABCG1	0,18	22,16	155,9	3,32	-	-	-	1
ABCG2	0,53	0,49	2,88	1,1	1,08	0,52	0,58	1
ABCG4	0,06	1,97	1,64	8,26	5,04	1,57	1,04	1
ABCG5	-	-	-	-	-	-	-	-
ABCG8	-	-	-	-	-	-	-	-
TAP1	0,09	0,64	0,04	0,29	0,08	0,11	1,42	1
TAP2	0,28	1,58	0,05	0,7	0,24	0,27	0,89	1

[1]: calibrator; [2]: RQ values calculated using the equation RQ=2-ΔΔ C_t. ΔΔC_t values were calculated by subtracting the ΔC_t of the calibrator from the ΔC_t value of each target; [3]: Absent genes defined as genes with ΔC_t above 25 cycles. 0 = RQ< 1E-3.

Overall, the MeJR8 cells appeared to be characterized by a modest modulation of transporter genes, and several transporters were already up-regulated in adherent cells. However, an interesting pattern of modulation was observed in the Me14346 system in which several transporters (ABCA2-3-5-7-8-9-10-11-13; ABCB5-7-8-10; ABCC1-2-3-5-6-8-11; ABCD4; ABCE1; ABCF1-2-3; ABCG1-4 and TAP2) displayed increased expression in 14346S melanospheres as compared to both the corresponding adherent (Me14346ADH) cells and melanocytes (Figure 4.4).

Figure 4.4. Differential expression of ABC transporters by Me14346S or Me14346ADH cells. Several members of ABC transporter families are differentially expressed on Me14346S (grey columns) with respect to their adherent counterpart (Me14346ADH, black columns). RQ values (calculated as indicated in Table 4.4) are reported on Y axes in logarithmic scale. Melanocytes were used as calibrator.

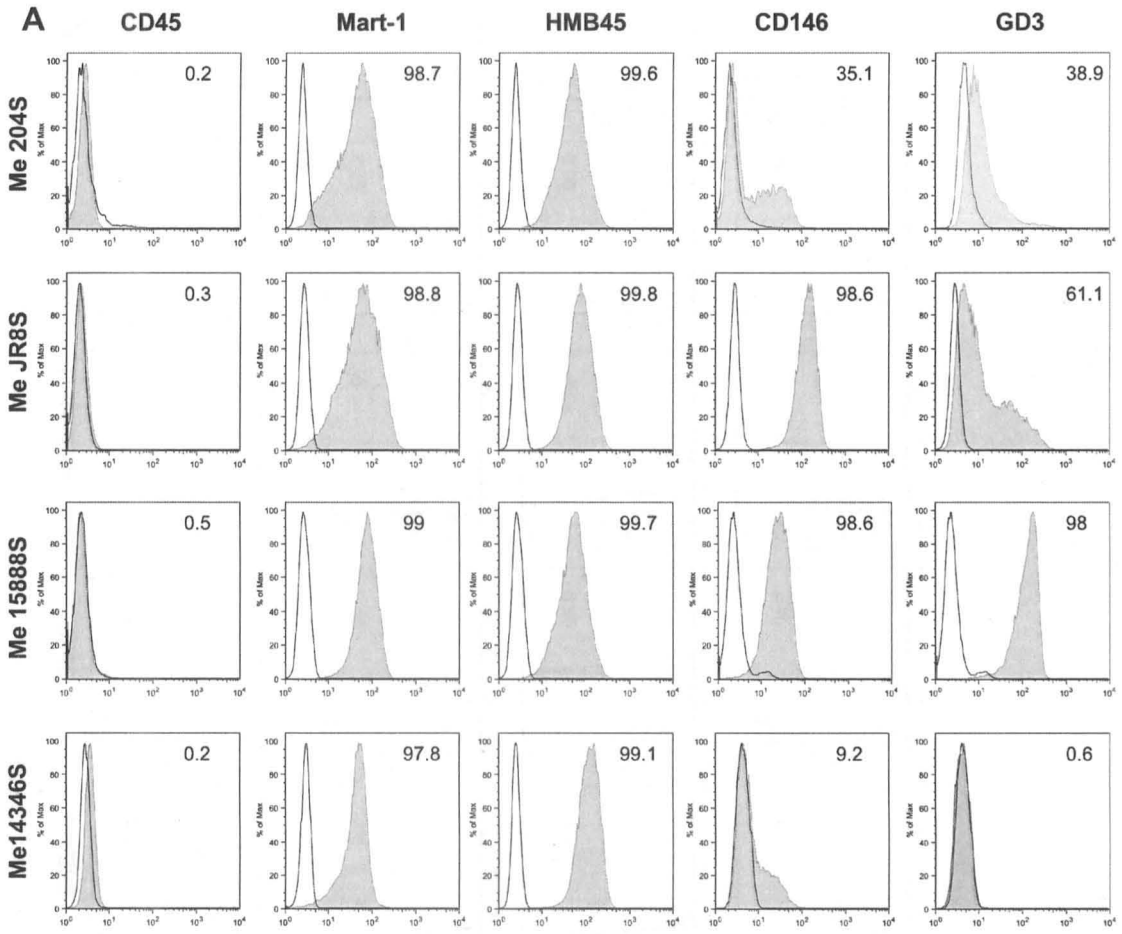


4.2.3 Expression of melanoma differentiation and stem-related markers on melanospheres and adherent cells

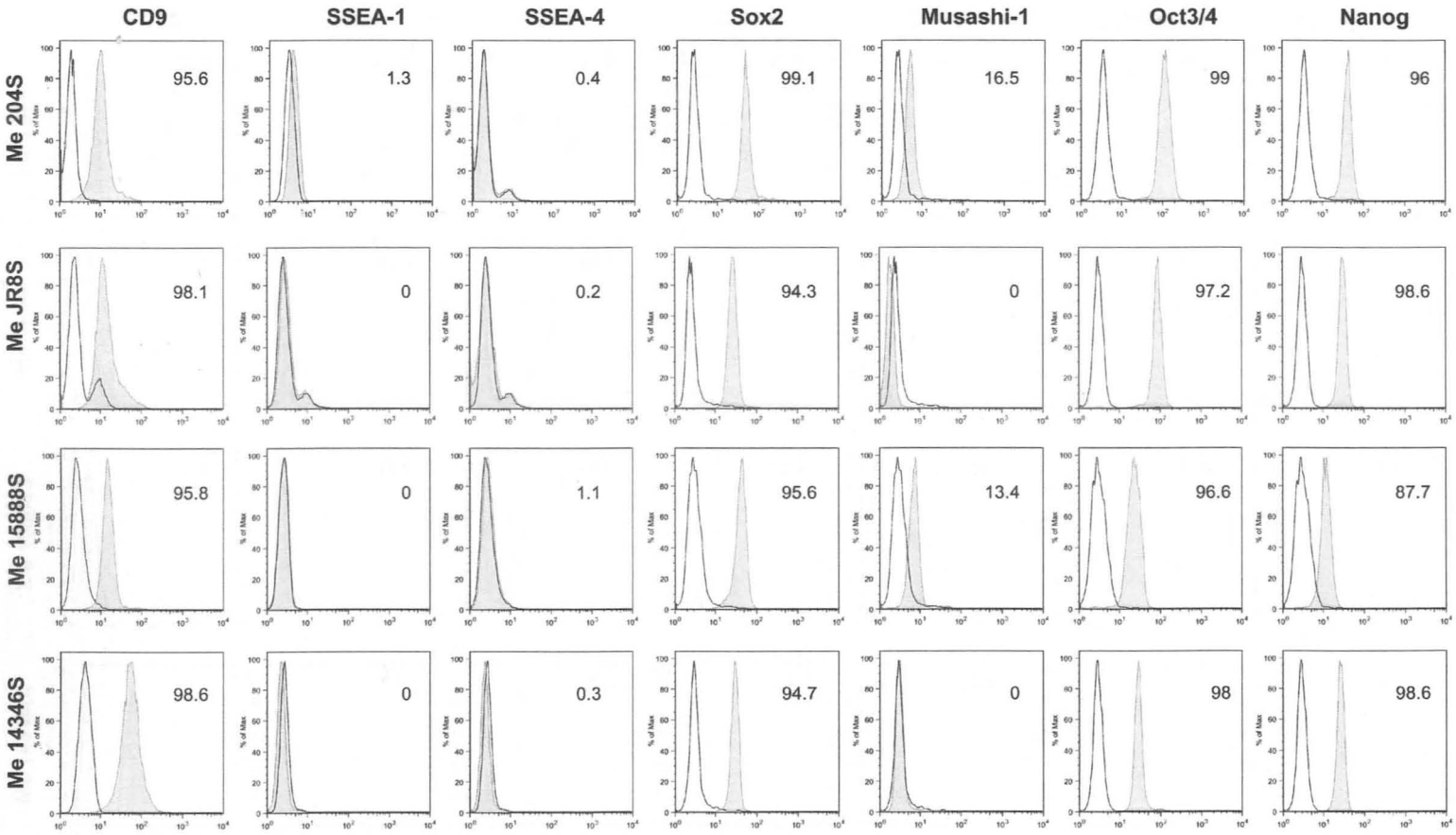
All the generated melanospheres were negative for the haematopoietic marker CD45, while they displayed 100% positivity for MelanA/Mart-1 and HMB45 melanocyte differentiation proteins (Figure 4.5A). The expression of the melanoma associated markers CD146 and GD3 was variable with virtually all Me14346S cells staining negative for GD3 and a very low percentage staining positive for CD146 (Figure 4.5A). Melanosphere cells did also express several embryonic stem cells markers and all the melanospheres behaved similarly being nearly 100% positive for CD9, Sox2 Oct3/4 and Nanog proteins, whereas a very low percentage of cells (or none at all) expressed SSEA-1 and SSEA-4. A low rate of expression was observed for Musashi-1, with 13-16% Me15888S and Me204S staining positive (Figure 4.5B).

The expression of stem cell-related markers (CD20, CD24, CD44, CD73, CD133, CD166, NGFR, Nestin) identifying adult stem cells of normal and cancer tissues (O'Brien et al, 2009) was examined. CD73 and CD166 were also included because of their expression by mesenchymal stem cells, while NGFR and Nestin for their association to cells of the neural crest from which melanocytes originate (Lee et al, 2007). All the melanospheres were negative for CD20 and highly positive for CD44 and Nestin. Conversely CD24, CD73, CD166, NGFR and CD133 markers were differently expressed in melanospheres lines. In Me15888S the large majority of cells were $CD133^+$, $CD166^+$ and $CD73^+$ and these melanospheres displayed the highest percentage of NGFR positive cells (Figure 4.5C).

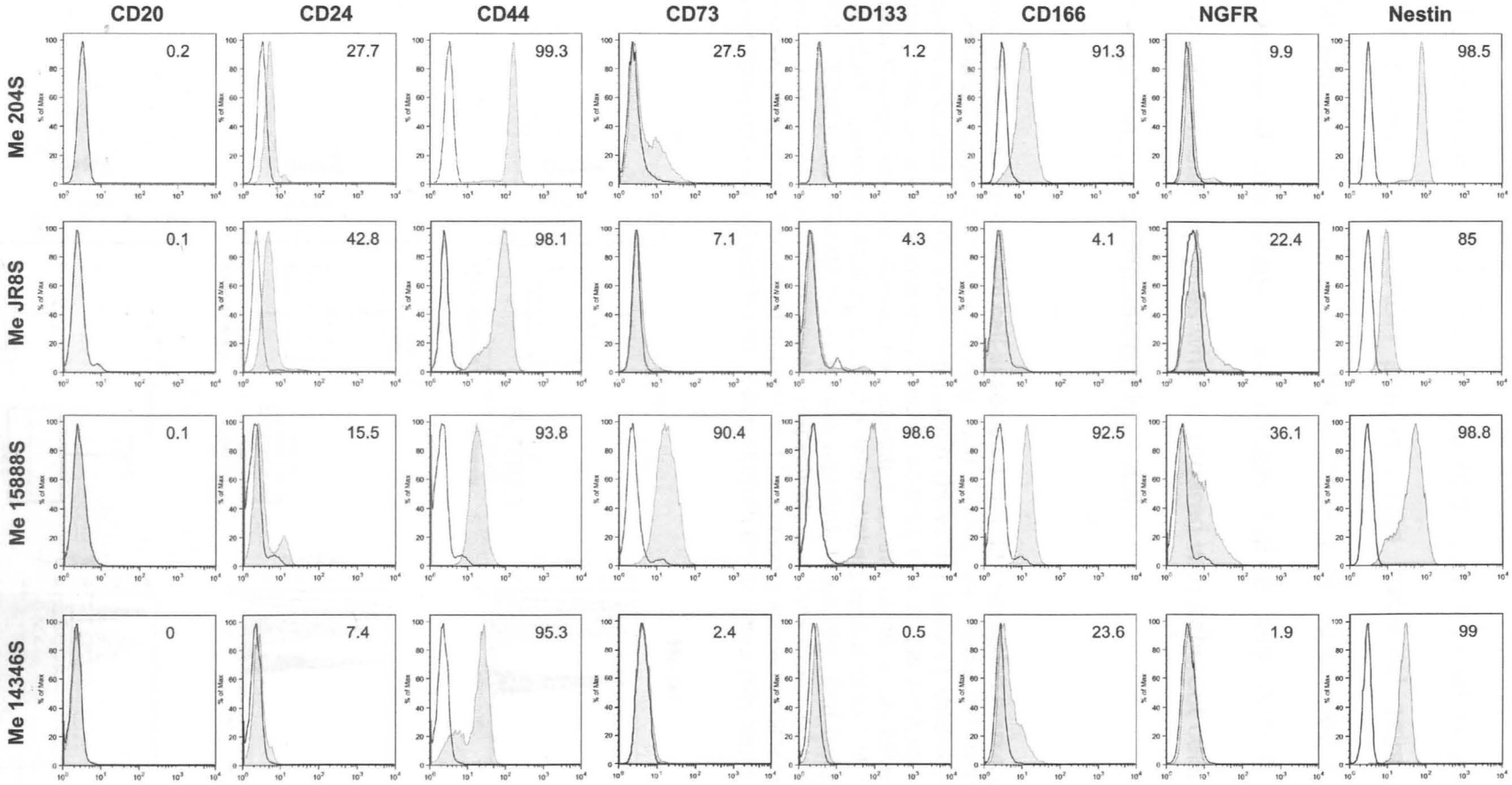
Figure 4.5. Immunophenotype of melanospheres. FACS analysis for the expression of CD45 and melanoma associated markers Melan-A/Mart-1; HMB45, CD146, GD3 (**A**), embryonic stem cell associated markers CD9, SSEA-1, SSEA-4, Sox2, Musashi-1, Oct3/4, Nanog (**B**) and adult stem cell related markers CD20, CD24, CD44, CD73, CD133, CD166, NGFR and Nestin (**C**). Thin black histograms represent controls stained with negative isotype antibodies; filled histograms correspond to specific markers; numbers indicate the percentage of positive cells. Histograms are representative of three independent experiments.



B

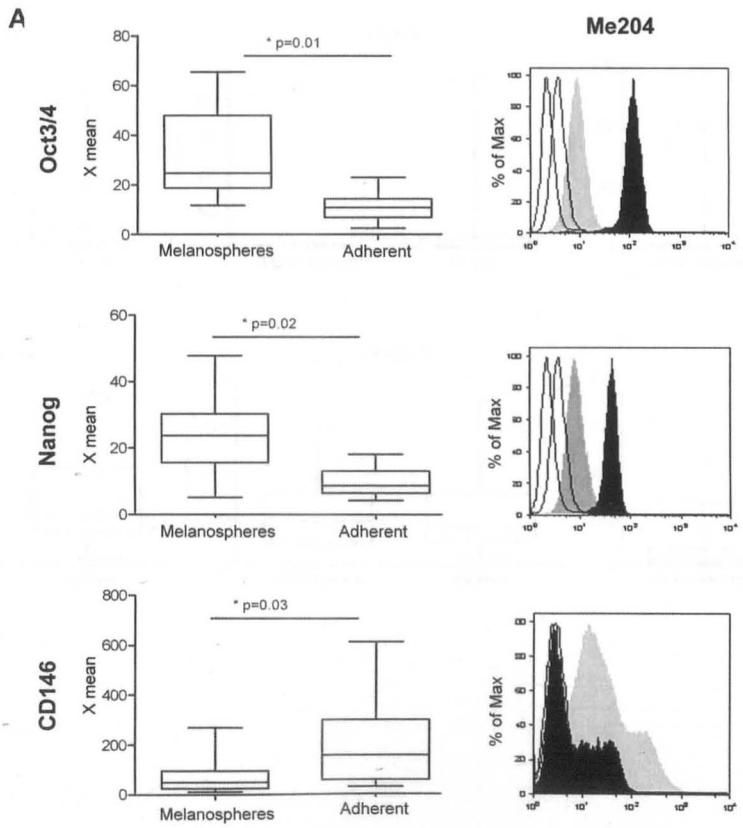

CD9
SSEA-1
SSEA-4
Sox2
Musashi-1
Oct3/4
Nanog
Me 204S
95.6
1.3
0.4
99.1
16.5
99
96
Me JR8S
98.1
0
0.2
94.3
0
97.2
98.6
Me 15888S
95.8
0
1.1
95.6
13.4
96.6
87.7
Me 14346S
98.6
0
0.3
94.7
0
98
98.6

C

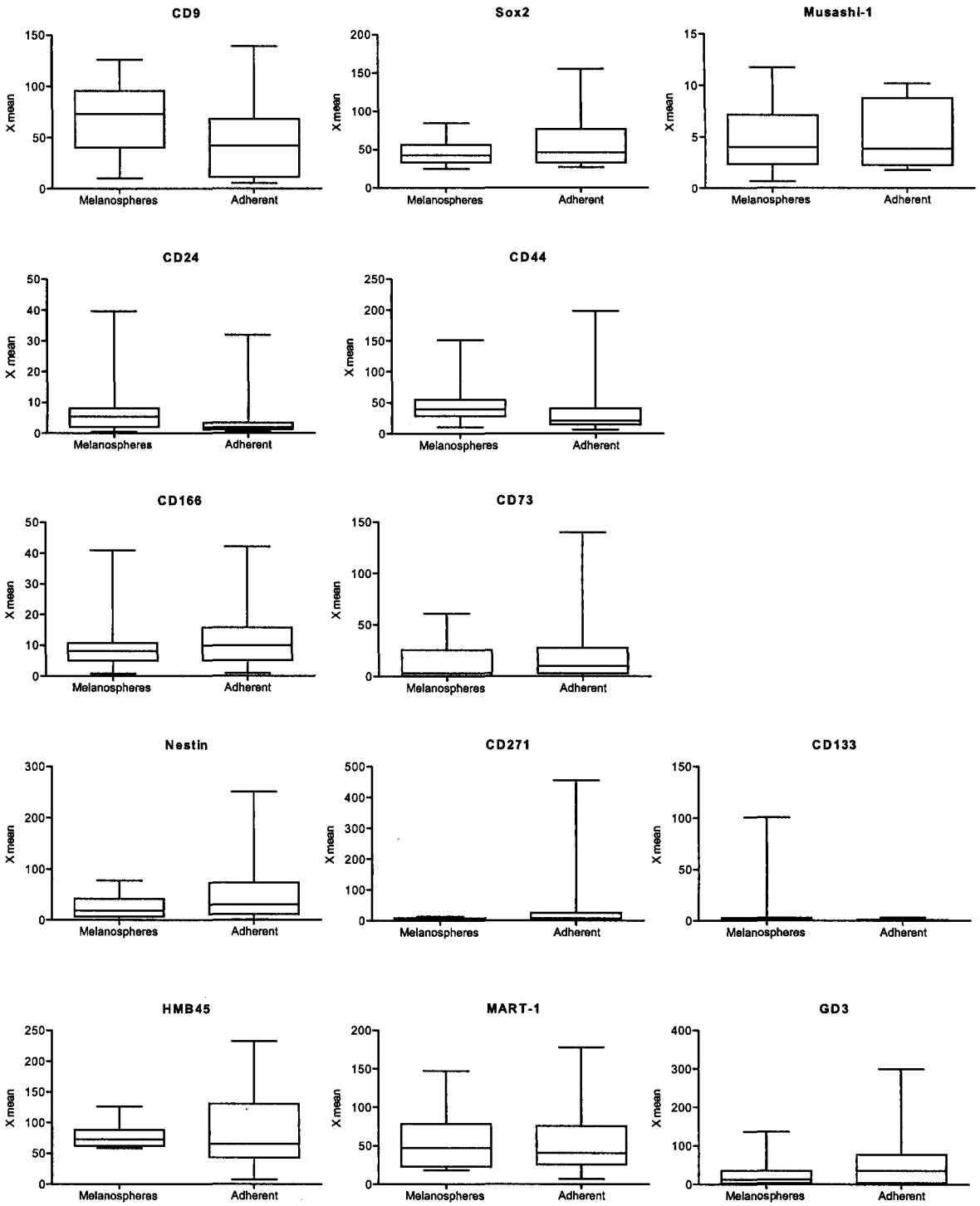

CD20
CD24
CD44
CD73
CD133
CD166
NGFR
Nestin
Me 204S
Me JR8S
Me 15888S
Me 14346S
0.2
27.7
99.3
27.5
1.2
91.3
9.9
98.5
0.1
42.8
98.1
7.1
4.3
4.1
22.4
85
0.1
15.5
93.8
90.4
98.6
92.5
36.1
98.8
0
7.4
95.3
2.4
0.5
23.6
1.9
99
% of Max

The expression of the above markers was also assessed in Me204ADH, JR8ADH, and Me14346ADH and in seven additional pairs of adherent and melanosphere melanoma cell lines. Among the examined markers, only the expression of the stem-related protein Nanog and Oct3-4 was significantly enhanced in melanospheres when compared with adherent cells, and only CD146 was significantly downregulated (Figure 4.6)

Figure 4.6 Expression of stem cell-associated and differentiation markers on melanospheres and adherent cells. **A**) Oct3/4, Nanog, and CD146 are modulated in melanospheres when compared with adherent cells. The left column shows the box-and-whisker plots for the indicated markers. The fluorescence intensity (X mean) on the y axis equals the X mean (marker) minus the X mean (isotype control). Values are calculated as the mean ± SE (Standard Error) of three independent experiments. Histograms of the differential expression of Oct3/4, Nanog, and CD146 in Me204S (black) and Me204ADH (gray) are reported. Lines and filled histograms correspond to isotype controls and specific antibodies, respectively (p-values evaluated with unpaired t-test). **B)** Box-and-whisker plots of analyzed markers in 11 melanoma adherent cell lines and melanospheres whose expression was not significantly different on melanospheres with respect to adherent cells.

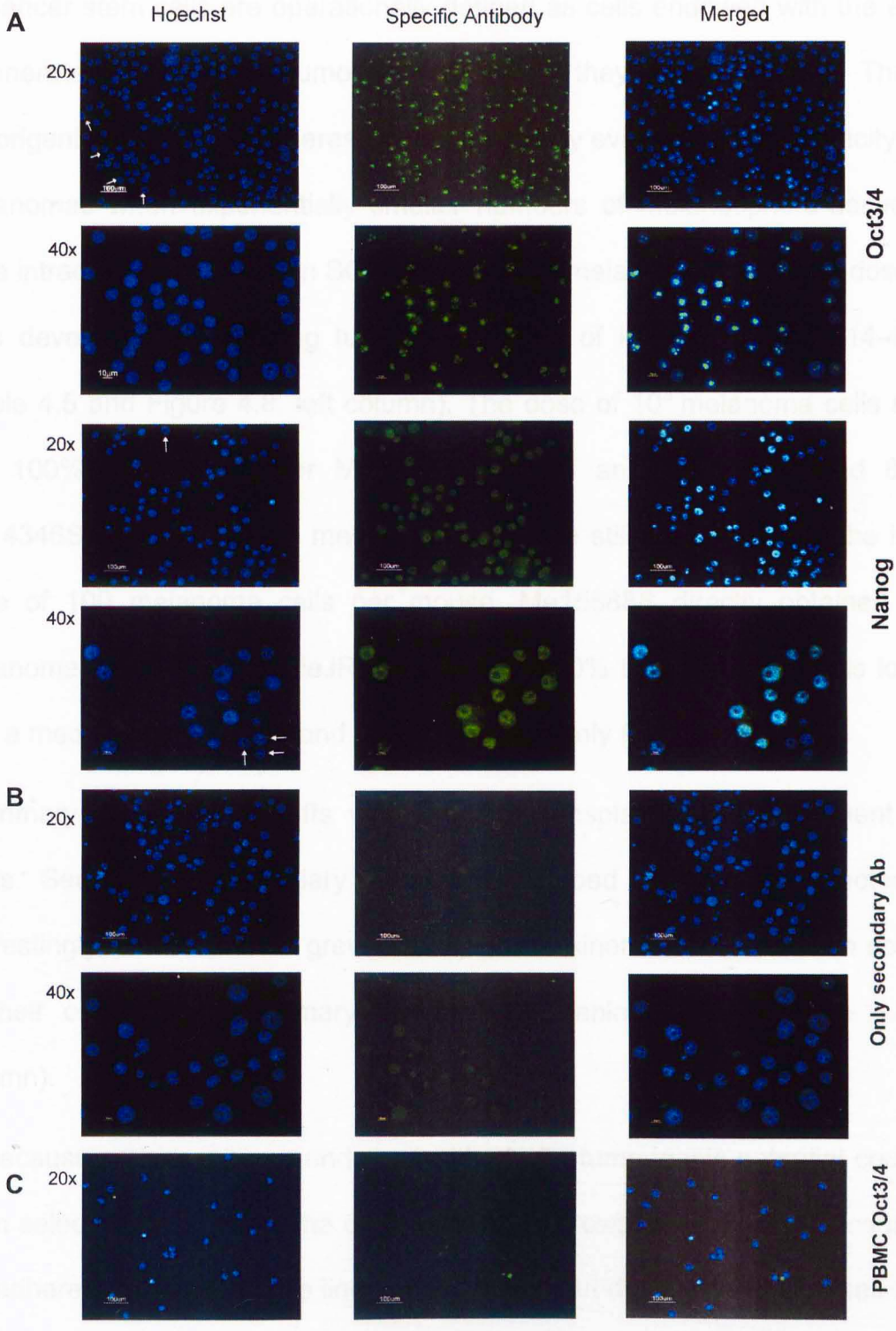


B


CD9
X mean
150
100
50
0
Melanospheres
Adherent
Sox2
X mean
200
150
100
50
0
Melanospheres
Adherent
Musashi-1
X mean
15
10
5
0
Melanospheres
Adherent
CD24
X mean
50
40
30
20
10
0
Melanospheres
Adherent
CD44
X mean
250
200
150
100
50
0
Melanospheres
Adherent
CD166
X mean
50
40
30
20
10
0
Melanospheres
Adherent
CD73
X mean
150
100
50
0
Melanospheres
Adherent
Nestin
X mean
300
200
100
0
Melanospheres
Adherent
CD271
X mean
500
400
300
200
100
0
Melanospheres
Adherent
CD133
X mean
150
100
50
0
Melanospheres
Adherent
HMB45
X mean
250
200
150
100
50
0
Melanospheres
Adherent
MART-1
X mean
200
150
100
50
0
Melanospheres
Adherent
GD3
X mean
400
300
200
100
0
Melanospheres
Adherent

Expression of Nanog and Oct3/4 was further analyzed by immunofluorescence, confirming the nuclear localization and the specific expression pattern of these embryonic proteins on melanosphere-derived cells (Figure 4.7).

Figure 4.7 Expression of Oct3/4 and Nanog in Melanospheres. Me14346S were dissociated and 10^5 melanoma single cells seeded onto a microscope slide by cytospin. Staining was performed using Oct3/4 or Nanog antibodies (green, Alexa 488) and Hoechst33342 for nuclei counterstaining (A). Negative controls include staining omitting the primary antibody (B) and a complete staining on PBMC (Peripheral Blood Mononuclear Cell) (C). Images at 20X (scale bar 100μ) and 40X (scale bar 10μ) have been reported. Merge of blue and green images showed that all cells expressed Oct3/4 or Nanog in the nucleus. White arrows indicate the few cells not displaying any positivity for the examined markers.



4.3 Melanospheres are endowed with enhanced tumorigenicity as compared to adherent melanoma cells

Cancer stem cells are operationally defined as cells endowed with the ability to regenerate *in vivo* the tumour from which they were derived. Thus, the tumorigenicity of melanospheres was assessed by evaluating their capacity to form melanomas when exponentially smaller numbers of melanosphere-derived cells were intradermally injected in SCID mice. All the melanospheres at the dose of 10^4 cells developed fast-growing tumours in 100% of injected mice in 14-48 days (Table 4.5 and Figure 4.8, left column). The dose of 10^3 melanoma cells resulted into 100% tumour take for Me204S, MeJR8S and Me15888S and 80% for Me14346S. Of note, all the melanospheres were still tumorigenic at the injecting dose of 100 melanoma cells per mouse. Me15888S directly obtained from a melanoma specimen and MeJR8S displayed 100% tumour take at this low dose with a median latency of 53 and 22 days respectively (Table 4.5).

Primary tumour xenografts were serially transplanted in subsequent animal hosts. Secondary and tertiary tumours developed in 100% of injected mice; interestingly, serial tumours grew with the same kinetics or faster when compared to their corresponding primary engraftment (Table 4.5 and Figure 4.8, right column).

Because melanospheres endowed with strong tumorigenic potential could have been selected from melanoma cells adapted to growth in FCS, and because even the adherent melanoma cells lines had a limited but detectable *in vitro* self-renewal

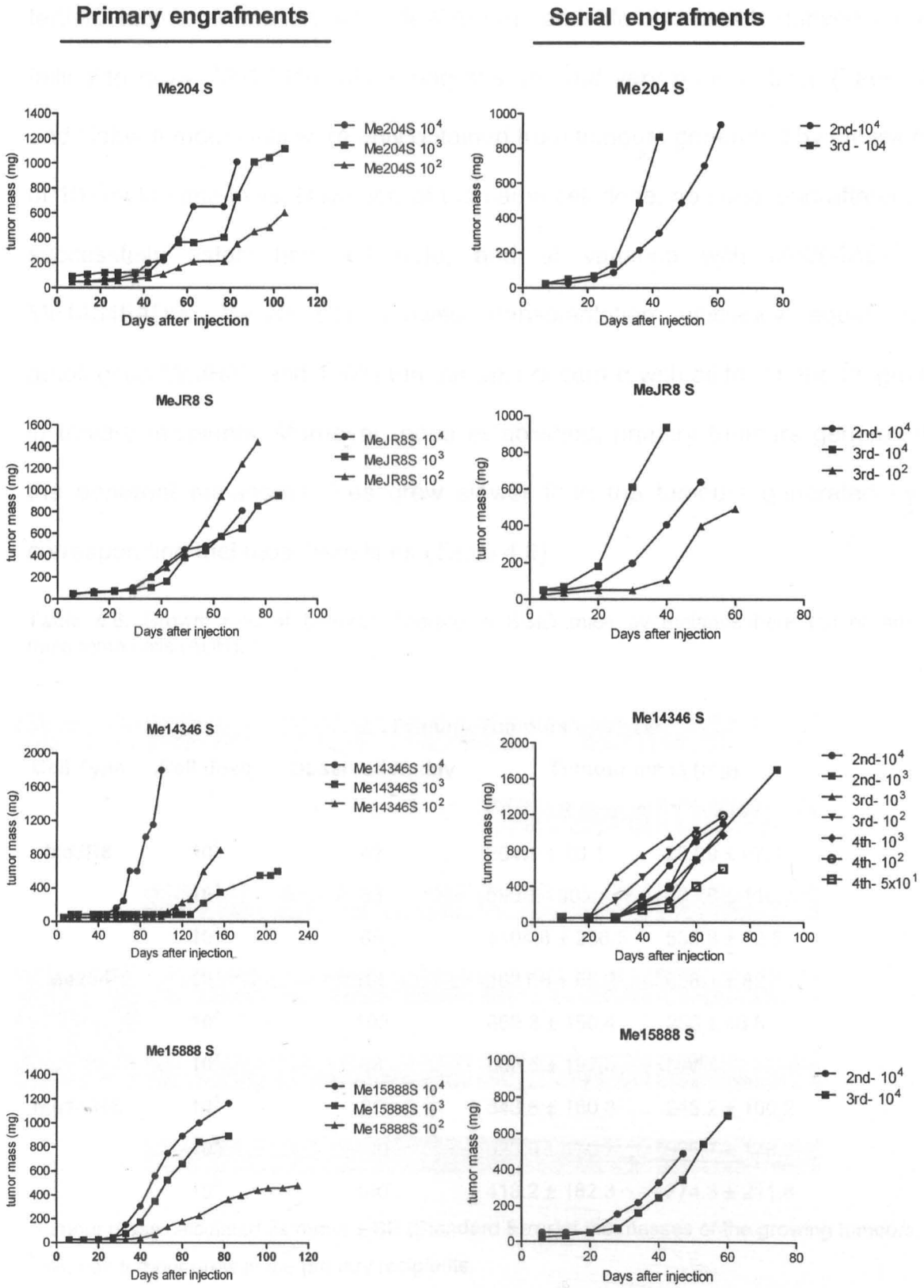
capacity, Me204ADH, MeJR8ADH and Me14346ADH were inoculated in SCID mice to evaluate the extent to which they possess tumour-initiating capacity *in vivo*. As expected, tumour take decreased with diminishing numbers of injected cells; the efficiency of primary tumour formation was lower when compared with that of corresponding melanospheres and the latency of tumour formation was longer (Table 4.5).

Table 4.5 *In vivo* tumorigenicity of melanosphere forming cells (S) and adherent melanoma cells (ADH).

Cell Type		Cell number[1]	Primary tumour formation		Secondary tumour formation		Tertiary tumour formation	
			Take[2]	*Latency*[3]	*Take*[2]	*Latency*[3]	*Take*[2]	*Latency*[3]
MeJR8	*S*	10^4	5/5	22	2/2	25	2/2	18
		10^3	5/5	29	ND	ND	ND	ND
		10^2	5/5	22	ND	ND	2/2	35
	ADH	10^4	5/5	22	2/2	25	2/2	30
		10^3	4/5	49	ND	ND	ND	ND
		10^2	3/5	49	ND	ND	2/2	50
Me204	*S*	10^4	4/4	14	2/2	36	2/2	28
		10^3	5/5	22	ND	ND	ND	ND
		10^2	2/5	39	ND	ND	ND	ND
	ADH	10^5	ND	ND	2/2	36	0/2	ND
		10^4	5/5	25	2/2	60	0/2	ND
		10^3	5/5	67	ND	ND	ND	ND
		10^2	0/5	ND	ND	ND	ND	ND
Me14346	*S*	10^4	5/5	48	2/2	30	ND	ND
		10^3	4/5	92	3/3	40	2/2	30
		10^2	3/5	123	3/3	40	3/3	40
	ADH	$5x10^1$	ND	ND	ND	ND	3/3	57
		10^6	2/2	19	0/1[4]	ND	ND	ND
		10^4	5/5	77	0/2[4]	ND	ND	ND
		10^3	4/5	77	ND	ND	ND	ND
		10^2	3/5	123	0/2[4]	ND	ND	ND
Me15888S	*S*	10^4	5/5	33	3/3	35	3/3	35
		10^3	5/5	33	ND	ND	ND	ND
		10^2	5/5	53	ND	ND	ND	ND

[1] Number of injected cells; [2] Number of tumours formed per number of injection; [3] Time after injection to tumour measurability; ND = Not Done. Median values are reported. Observation time is 200 days.[4] Observation time is 60 days.

Figure 4.8. Tumorigenic potential of melanospheres. Growth curves (GC) of primary (left) or serially transplanted (right) human melanoma xenografts generated by intradermal injection of different doses of melanosphere-derived cells. Tumour mass reported on the Y axis is expressed in mg and calculated as the mean of the masses of the growing tumours. For details on the number of mice treated with each inoculating dose, see Table 4.5.



Primary tumour derived from adherent cells were serially transplanted in additional recipients. Differences in transplantability, evaluated as tumour take, between adherent and melanosphere-derived primary xenograft clearly emerged in tertiary tumour formation with Me204ADH- and Me13436ADH- derived tumours failing to grow. Me14346ADH xenografts showed very poor viability (Table 4.5), and viable tumour cells were only obtained from tumours generated by the injection of 10^6 melanoma cells. However, at the same cell dose, no serial engraftment was successfully established. Of note, and at variance with Me204ADH and Me14346ADH, MeJR8ADH showed transplantation efficiency equal to its autologous MeJR8S and 100% tumour take occurred with all four tumours growing in tertiary recipients. Moreover, once established, primary tumours generated by the adherent melanoma lines grew slower than the tumours generated by the corresponding melanosphere lines (Table 4.6).

Table 4.6. Growth rate of tumors generate in SCID mice by melanosphere (S) or adherent melanoma cells (ADH).

	Primary Tumours			
Cell Type	**Cell dose**	**Observation day**	**Tumour mass (mg)[1]**	
			S	*ADH*
MeJR8	10^4	42	264.1 ± 70.1	257.9 ± 67.1
	10^3	63	590 ± 130.4	363.8 ± 110.7
	10^2	85	1104.8 ± 256.5	538.8 ± 82.5
Me204	10^4	105	1021.8 ± 65.2	638.1 ± 82
	10^3	105	869.3 ± 150.4	256 ± 48.6
	10^2	83	602.5 ± 197.5	NA[2]
Me14346	10^4	85	643.5 ± 160.8	248.2 ± 100.2
	10^3	120	523.9 ± 170.7	926.7 ± 178.3
	10^2	140	418.2 ± 182.3	274.3 ± 211.8

[1] tumour mass calculated as mean ± SE (Standard Error)of the masses of the growing tumours.

[2] NA, non tumour grew in the primary recipients.

These quantitative differences in the *in vivo* behaviour of melanospheres and adherent melanoma cells were assessed by statistical analysis. The log-normal regression model yielded highly significant results for treatment (adherent vs melanosphere cells; $p<0.0001$), thus confirming the higher tumorigenic potential of melanospheres when compared with adherent melanoma cells. Statistically significant results were also obtained for cell line ($p<0.0001$) and dose ($p<0.0001$), indicating that both factors affected tumorigenicity. Conversely, the interactions between treatment and cell line ($p=0.22$) or inoculating dose ($P=0.30$) were not significant, demonstrating that melanospheres have higher tumorigenic potential that adherent melanoma cells, independently of cell line and dose. Tumour mass was significantly affected by treatment (adherent vs melanosphere cells; $p<=0.0045$) but not by cell line ($p=0.16$), and no significant interactions between treatment and cell line ($p=0.29$) or treatment and dose ($p=0.79$) were found (Table 4.6). These analyses support the conclusion that, once established, tumours generated by melanospheres grow more aggressively, and this aggressive behaviour is a function of their melanosphere origin.

Take together the *in vivo* results suggest that melanoma initiating cells can also be found in melanoma cell lines when grown as adherent cells, even if the frequency of self-renewing cells was relatively lower in adherent cells when compared to cells from melanospheres.

To provide evidence that the melanoma xenografts generated by melanospheres may have a clonal origin, SCID mice were also inoculated with escalating dose of cells derived from melanospheres generated from a single-cell melanoma clone, P1E8S. P1E8S showed a self-renewal efficiency (20.6% ± 5.1)

similar to that of the JR8S spheres from which this clone had been derived (Table 4.2). Tumour take, latency and serial tumour initiating capacity were all similar to those of its parental melanosphere bulk population (MeJR8S) (Table 4.7).

Table 4.7 *In vivo* multipotency of the melanosphere single cell-derived clone P1E8S.

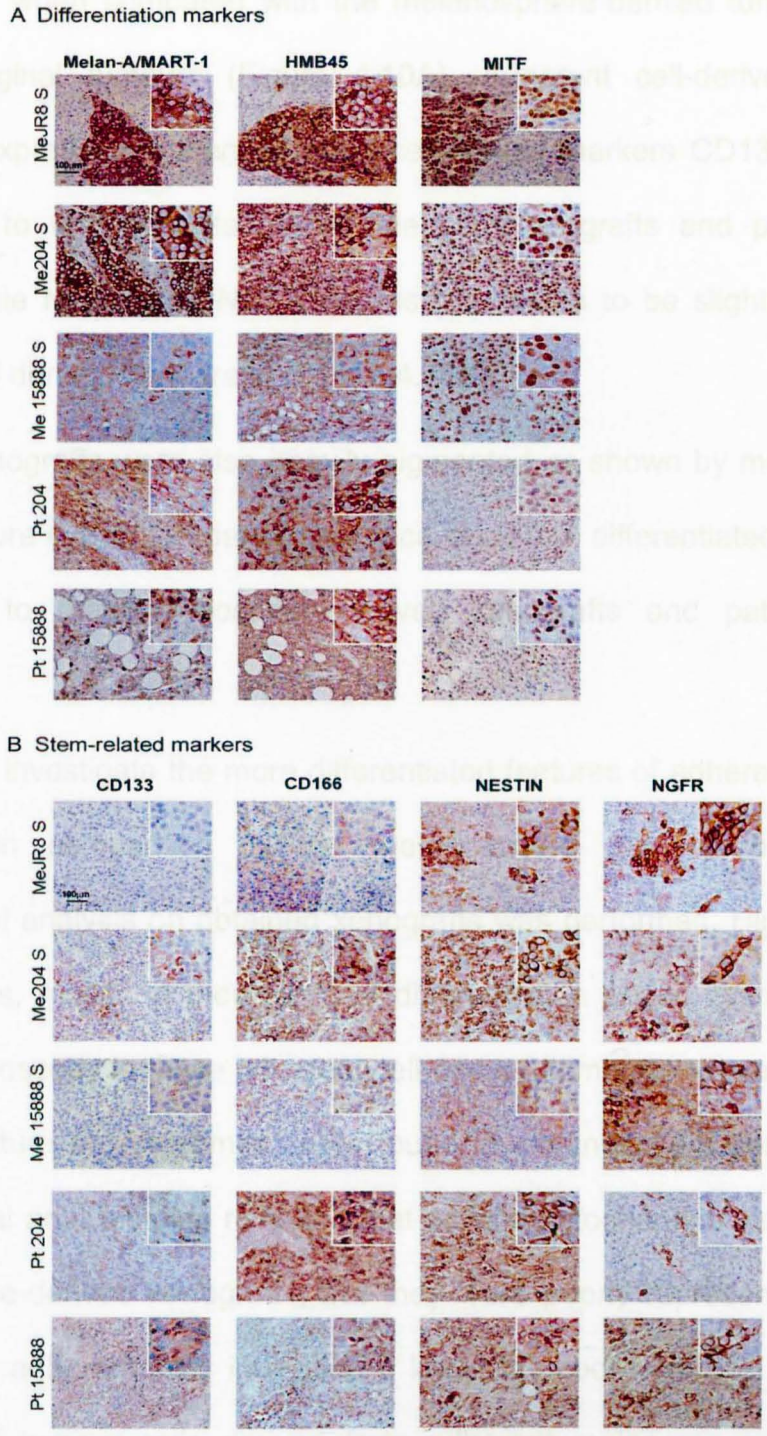
Cell type	Cell number[1]	Primary tumour formation		Secondary tumour formation	
		Take[2]	*Latency*[3]	*Take*[2]	*Latency*[3]
MeP1E8S	10^4	5/5	28	2/2	26
	10^3	5/5	28	ND[4]	
	10^2	5/5	39	2/2	26

[1]Number of injected cells; [2] Number of tumours formed per number of mice injectes; [3] Time after injection to tumour measurability; [4]ND: not done. Median values are reported. Observation time is 200 days.

4.4 Xenografts generated by melanospheres recapitulate the phenotypic features of the original human melanoma

Histological evaluation confirmed that human melanoma xenografts generated in SCID mice by melanospheres consist of cells showing the typical morphology of melanoma cells: eosinophilic cytoplasm, irregular nuclei and prominent nucleoli. Xenografted tumours expressed the specific melanoma markers HMB45, Melan-A/Mart-1 and MITF-1. Moreover, although melanospheres were found homogeneously positive for Melan-A/Mart-1, this marker was heterogeneously expressed in the growing tumour mass, i.e., Melan-A/MART-1-negative melanoma cells could be found. In general, this heterogeneity as well as the level of MITF and HMB45 mirrored that of the patient's original tumour (Figure 4.9A). The similarity between melanospheres-derived tumours and autologous patients' tumours was also maintained with regard to the expression of the stem cell-related markers CD133, CD166, NGFR and Nestin (Figure 4.9B). The stem cell-related markers showed an elevated degree of variability in expression, with CD166 and Nestin expressed by the majority of cells in the tumour mass, while NGFR is found in a minority of clustered cells and CD133 was displayed only by very rare cells.

Figure 4.9. Melanospheres reproduce human melanoma in immunocompromised mice. Immunohistological staining of (**A**) melanoma-associated and (**B**) stem cell-related markers in xenograft tumours generated by intradermal injection of melanosphere-derived cells and in lymph node metastatic melanomas of patient 204 and patient 15888. Internal scale markers are indicated in each panel. For melanosphere-derived xenografts, reported images are representative of eight independently analyzed xenografts; for patients' tumours, images are representative of two non-consecutive tumour sections. Scale bar =100 um.



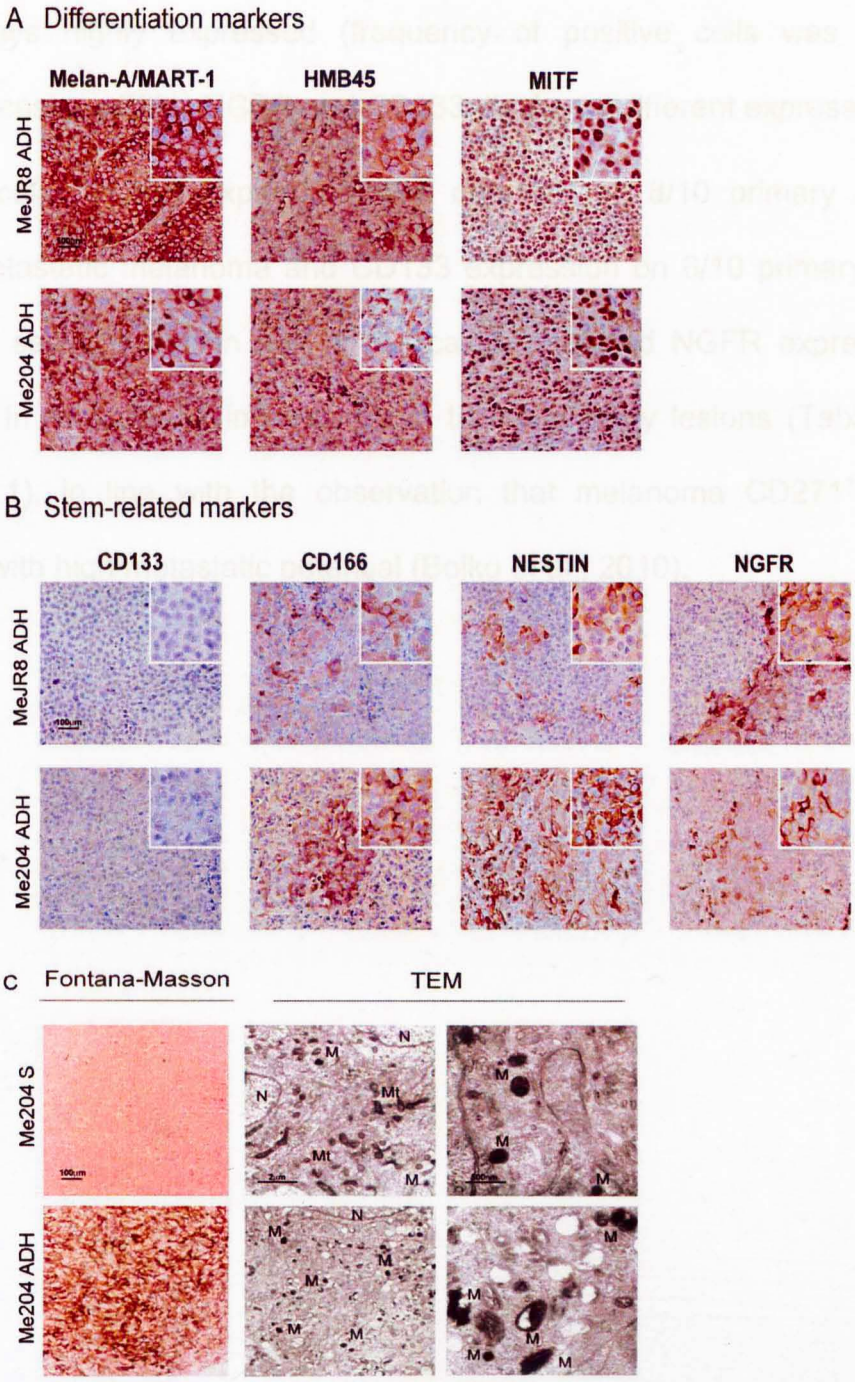
Conversely, immunohistochemistry on adherent cell-derived xenografts, showed that the differentiation markers Melan-A/MART-1 and HMB45 are homogenously expressed at high level and MITF transcription factor expression is enhanced when compared with the melanosphere-derived tumours and the patient's original tumours (Figure 4.10A). Adherent cell-derived xenografts display an expression pattern of stem cell-related markers CD133 and CD166 comparable to that of melanosphere-derived xenografts and patient original tumours, while Nestin and NGFR expression seems to be slightly reduced on adherent-cell derived xenografts (Figure 4.10B).

These xenografts were also heavily pigmented as shown by melanin specific staining (Figure 4.10C, left panel) evidence of a more differentiated phenotype in comparison to the melanosphere-derived xenografts and patients' original tumours.

To further investigate the more differentiated features of adherent cell-derived xenografts in comparison to the melanosphere- derived ones, electron microscopical analysis on obtained xenografts was performed. High numbers of melanosomes, mostly in their terminal differentiation phase, were detected by electron microscopy in these adherent cell-derived tumours, whereas only a few, mainly immature melanosomes, were found in melanosphere-derived tumours. Ultrastructural analysis also revealed that several mitochondria were present on melanosphere-derived xenografts, but they were poorly represented in tumour derived from adherent cells indicating a lower metabolic activity. Finally, in the cytoplasm of tumour cells derived from adherent melanoma cell lines, large

vacuoles compatible with on-going autophagic processes were frequently observed. This ultra structural analysis confirms the observation that a low number of viable cells could have been recovered after mechanical dissociation of xenografts generated by adherent melanoma cells (Figure 4.10C, right panel).

Figure 4.10. Immunohistochemistry and ultrastructural analysis of xenografted tumours. Immunohistochemical staining of (**A**) melanoma-associated and (**B**) stem cell-related markers was performed on xenografts generated by intradermal injection of adherent melanoma cells Me204 and MeJR8. For each cell line, reported images are representative of eight independently analyzed xenografts. Internal scale markers are indicated in each panel. (**C**) Melanin staining and transmission electron microscopy (TEM) analysis. Fontana-Masson melanin-specific staining (**C**, left panels) and TEM (**C**, middle and right panels) on melanoma xenografts derived from the injection of Me204 melanospheres (upper panels) or Me204-adherent cells (lower panels). M, melanosomes; Mt, mitochondria; N, nucleus. Scale bar=100 um. For TEM, left panel scale bar =2 um and right panel scale bar=500nm.



4.5 Expression of stem cell-related markers in human melanoma

The analysis of the expression of stem cell-related markers was extended to 10 primary melanoma lesions and their paired metastases. While and Nestin were always highly expressed (frequency of positive cells was in all the examined cases > 70%) NGFR and CD133 displayed different expression levels.

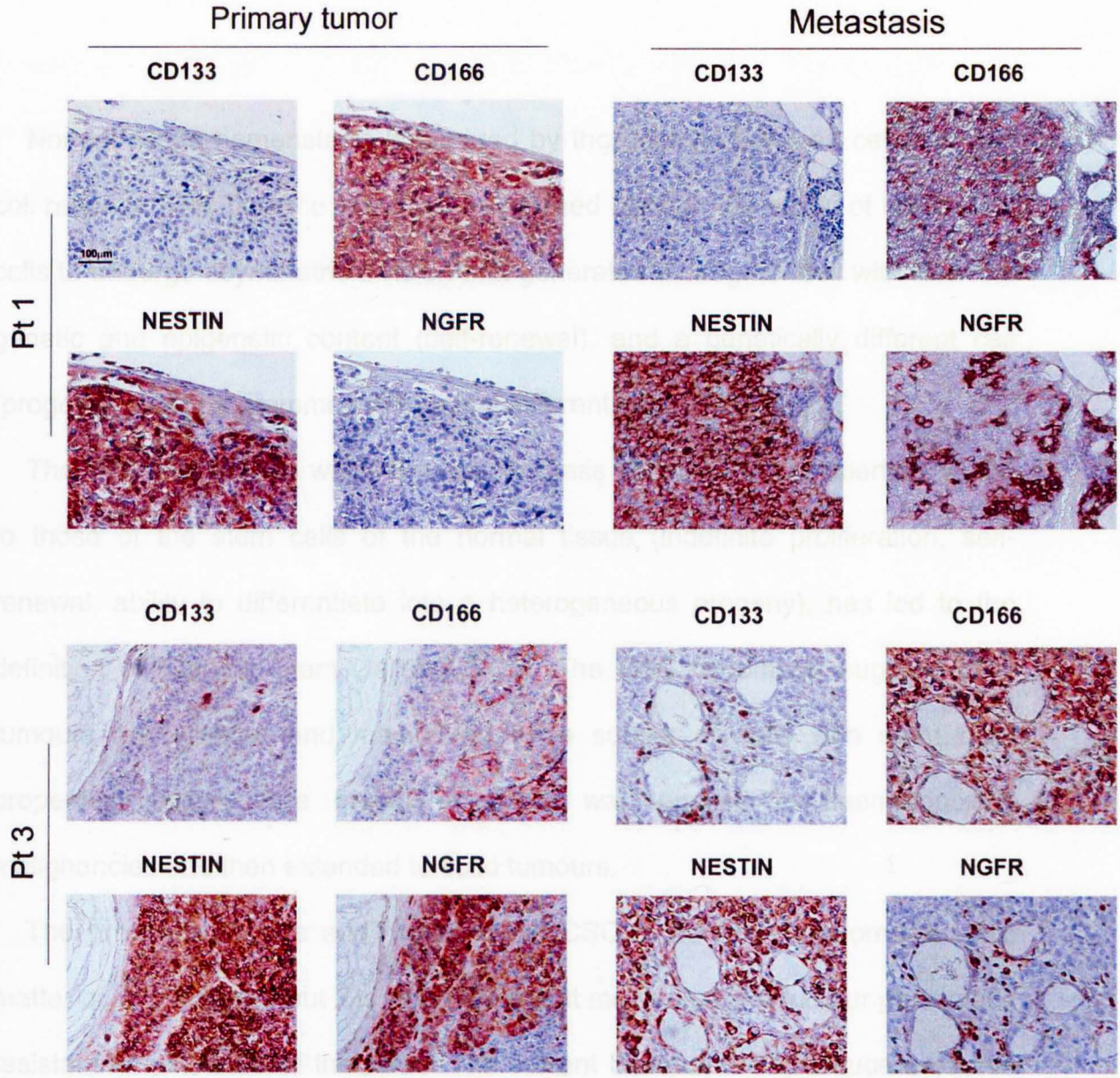
In particular, NGFR expression was detected on 8/10 primary and 10/11 related metastatic melanoma and CD133 expression on 6/10 primary and 4/11 metastatic samples and in half of the cases analyzed NGFR expression was enhanced in metastases in comparison to the primary lesions (Table 4.8 and Figure 4.11), in line with the observation that melanoma $CD271^+$ cells are endowed with high metastatic potential (Boiko et al., 2010).

Table 4.8 Expression of stem cell- related markers on paired primary and metastatic melanoma.

				Primary tumours			
Patient		**NGFR**	**CD133**	**Clark Level**	**Thickness (mm)**	**Ulceration**	**Metastasis site**
1	P	-	-	IV	19	Yes	
	M	**	-				Lymph node
2	P	*	*	IV	5,3	No	
	M	**	-				Lymph node
	M	*	-				Soft tissues
3	P	*	*	IV	9,6	Yes	
	M	*	*				Lymph node
4	P	*	-	V	7,5	Yes	
	M	**	*				Lymph node
5	P	*	*	IV	4,9	Yes	
	M	**	-				Lymph node
6	P	*	*	IV	12	Yes	
	M	*	-				Lymph node
7	P	-	-	III	8	No	
	M	-	-				Lymph node
8	P	**	-	III	3,6	No	
	M	**	-				Soft tissues
9	P	**	*	IV	8,2	Yes	
	M	**	**				Soft tissues
10	P	**	*	IV	>4	Yes	
	M	**	**				Soft tissues

P: Primary tumours; M: Metastases; -:<5% of positive cells; *: <20% of positive cells; **: <70% of positive cells, ***: >70% of positive cells. Positive cells were evaluated in 10 random fields.

Figure 4.11. Expression level of stem-related markers differs between human melanomas. Immunohistological evaluation of the expression of the indicated stem cell-related markers in primary (left) and paired metastatic (right) melanomas. Reported images are representative of two independently analyzed tumour sections. Internal scale markers are indicated in each panel. The majority of tumour cells express (Pt3) CD166 and Nestin; rare CD133-positive cells could be found within the tumour mass in both primary and metastatic tumours (Pt3). (Pt1) NGFR is heterogeneously expressed, and in some cases enhanced, in metastatic lesions. Reported images are representative examples of ten primary and metastatic melanomas. Pt= Patient. Scale bar = 100 μm.



CHAPTER 5: DISCUSSION

Normal tissue homeostasis is assured by the balance between cell loss and cell renewal. This balance is indeed maintained through the ability of adult stem cells to undergo asymmetric division that generates a daughter cell with identical genetic and epigenetic content (self-renewal), and a genetically different cell (progenitor cell) programmed to terminal differentiation.

The discovery of cells within the tumour mass endowed with properties similar to those of the stem cells of the normal tissue (indefinite proliferation, self-renewal, ability to differentiate into a heterogeneous progeny), has led to the definition of "Cancer Stem Cells" (CSCs). The CSC hypothesis suggests that tumours are initiated and maintained by a subset of cells with stem cells properties. Initially, the model of CSCs was applied to haematopoietic malignancies and then extended to solid tumours.

The presence, nature and frequency of CSC in human melanoma are still matter of investigation, but it is well known that melanoma is a tumour particularly resistant to conventional therapies. The current treatments can induce objective tumour regression only in small cohort of patients, and these responses are not durable and often not associated with improved long-term survival. In this

perspective, the identification of melanoma cells endowed with strict tumour initiating capacity is considered a big challenge for the implications this finding may have in the understanding of melanoma biology and in the development of more effective treatments.

Two different approaches have been undertaken to show that human tumours contain a subpopulation of CSCs. One of these approaches directly sorted out putative candidate CSCs from the whole tumour mass using a combination of cell surface markers and the selected populations are then assayed *in vivo* for their ability to sustain tumour growth. An alternative method to isolate CSCs is based on the ability of stem cells to form non –adherent colonies when seeded at low density in a suitable culture medium. In this thesis this second approach has been used to investigate the presence and the nature of putative cancer stem cells in metastatic melanoma.

This thesis reports evidence further supporting the notion that human melanomas do contain cells endowed with *in vitro* and *in vivo* features of CSCs. From lymph node metastatic lesions, melanoma cells were isolated that kept distinctive features of melanomas such as the expression of Melan-A/Mart-1 and HMB45, but displayed the three main properties of CSCs, namely self-renewing capacity, multipotency and strong tumorigenic potential in immunocompromised mice. Moreover, the human melanoma xenografts recapitulated the original heterogeneity of the patient's tumours.

The concept that CSCs should represent a rare subset in the tumour bulk has been recently revised and, using a syngeneic model for CSC definition, it has

been shown that mouse leukaemia may indeed display a higher frequency of tumour-initiating cells than previously thought (Kelly et al, 2007). Further observation pointed out that the frequency of CSCs in solid cancer could be highly variable depending on patient samples, on the histological features of the tumour itself, with relatively more undifferentiated tumours containing a high proportion of CSCs (Harris et al., 2008).

Data contained in this thesis are in line with the above reported observation. In fact, data reported here showed that melanospheres could be selected from bulk melanoma cells with high efficiency and that these cells displayed an elevated clonogenicity and a strong *in vivo* tumorigenic potential suggesting a high frequency of tumour initiating cells in melanosphere cultures (Table 4.2 and Figure 4.5). Thus, these results are also consistent with the recent findings of Morrison's studies (Quintana et al., 2008; Quintana et al., 2010) and sustain the notion that CSC may not be so rare at least in human melanoma.

All the melanoma samples studied in this thesis were derived from lymph nodes or visceral metastasis and it is conceivable that metastases may be naturally enriched with regard to tumorigenic cells (Croker and Allan, 2008; Chiang and Massague, 2008); in fact, cells undergoing an epithelial–mesenchymal transition and acquiring the ability to invade and metastasize are also endowed with tumorigenic properties (Mani et al., 2008; Gupta et al., 2009). It is tempting to speculate that metastases could be particularly enriched for tumorigenic cells and, because it is known that melanomas are generally highly metastatic and endowed with a high degree of plasticity (Hendrix et al., 2003;

Mourad-Zeidan et al., 2008), this theory could explain the high frequency of tumorigenic cells in this particular human cancer.

Melanospheres expressed melanoma markers Melan-A/MART-1and HMB45 thus confirming their melanocytic origin. Two melanospheres selected for this study (Me204S and Me14346S) had $BRAF^{V600E}$ mutations while the other two melanospheres (MeJR8S and Me15888S) were BRAF wild type (Table 4.3). In these melanomas, the $BRAF^{V600E}$ mutation was associated with functional CDKN2A, while the BRAF wild type correlated with CDKN2A homozygous deletion. These findings are in line with general distribution of CDKN2A biallelic deletion in metastatic melanoma, deletion reported in about 45% of cases (Conway et al., 2010, Grafstrom et al., 2005); melanospheres do not express PTEN proteins, and this is correlated with a enhanced level of activated AKT (pAKT) (Figure 4.3). Moreover, it has been recently reported that PTEN deletion is found in glioma initiating cells and it was related to proliferation and self-renewal of these cancer stem cells, independently from the expression of the gliomas stem cells surface CD133 (Cheng et al., 2010).

The most common cause of multidrug resistance (MDR) in human cancers is related to the expression and function of one or more ATP-binding cassette (ABC) transporters that efflux anticancer drugs from cells. Due to the intrinsic chemoresistance of stem cells it has been postulated that these transporters should also be amplified /over-expressed in cancer stem cells. Thus in this thesis the ABC transporter expression profiles of melanospheres and adherent cell lines were carefully monitored. Data collected here showed that all the melanoma cells

express a group of ABC transporters (ABCA5, ABCB2, ABCB6, ABCD3, ABCD4, ABCF1, ABCF2 and ABCF3) that were previously reported as detectable at similar levels on cells of melanocytic origin (Heimerl et al., 2007). Conversely, a subset of transporters (ABCB3, ABCB6, ABCC2, ABCC4) was found to be upregulated on melanospheres as compared to adherent melanoma cell lines and melanocytes (Table 4.4). ABCB5, proposed as a marker form melanoma initiating cells (Schatton et al., 2008) was only detected in Me14346 spheres and adherent cells, although at a lower level of expression than in melanocytes (Table 4.4 and Figure 4.7). These data are in agreement with the observation that ABCB5 reveals a melanocyte-specific high transcript level and it is expressed in most melanoma cell lines, (Heimerl et al., 2007, Chen et al., 2005). The expression of a broad range of ABC transporters on cells of melanocytic origin may be related to the intrinsic biological features of melanocytes that develop a physiological resistance to toxic melanin intermediates and metabolites that have to be rapidly extruded from the cells. Interestingly, a model has been recently proposed that associates the intrinsic multidrug resistance of melanoma cells to the presence of immature melanosomes (Chen et al., 2009). According to this model, in normal pigment-producing cells such as melanocytes, the ABC transporter system regulates the homeostasis of melanocytes by trapping cytotoxic melanin intermediates into sub-cellular organelles such as melanosomes. In particular, melanosomes in stage II-III of maturation are important for the maintenance of homeostasis in melanocytic cells and should be

particularly enriched in ABC transporters. These physiological features could thus explain the high intrinsic resistance of melanoma cells to cytotoxic drugs.

In this thesis, melanosphere phenotype was further investigated by analyzing the expression of an array of markers associated with both embryonic and adult normal stem cells. Melanospheres were uniformly positive for some embryonic stem cell markers (CD9, Sox2, Oct3/4, and Nanog) while they displayed heterogeneous expression for most of the adult normal and cancer stem cells associated markers analyzed (CD20, CD24, CD166, CD133, NGFR) (Figure 4.4). Melanosphere phenotype was compared to that of adherent cells. Eleven paired adherent melanoma cell lines and melanospheres were included in the analysis. These data confirmed on a larger set of samples that the differentiation marker CD146 was more highly expressed in adherent melanoma cells while the embryonic stem cell markers Oct3/4 and Nanog displayed an enhanced expression in melanospheres, further endorsing the notion that melanosphere most likely represent a more undifferentiated, stem-like counterpart of melanoma cells (Figure 4.5A). Data from the literature indicate that the expression of Oct3/4 and Nanog embryonic stem cell markers is indeed increased in cancer stem-like cells isolated from different human solid tumours such as glioblastoma (Tomuleasa et al., 2010), lung (Leung et al., 2010) and ovarian cancer (Guo et al., 2011); moreover a role of Oct3/4 in blocking breast CSC differentiation and in determining resistance to chemotherapeutic drugs has been recently proved (Sajithlal et al., 2010 Honoki et al., 2010). In addition, in human cancers the expression of embryonic stem cell markers was associated with tumour

progression and bad prognosis (Schoenhals et al., 2009 and Wang et al., 2009). Together all this evidence further supports the immature and aggressive nature of melanospheres.

Melanospheres displayed a strong *in vivo* tumorigenicity in SCID mice and this strong tumour initiating capacity was maintained over serial *in vivo* passages (Table 4.5 and Figure 4.8). However, tumorigenicity was not linked to the expression of a given stem cell-associated marker. Recently, studies have stress the role of CD133 as a cancer stem cell marker in a series of human solid tumours, including melanoma, (O'Brien et al, 2009; Monzani et al., 2007). More recently, NGFR (CD271) has been associated with melanoma initiating cells (Boiko et al., 2010). In the present thesis it is shown that Me15888S melanospheres were highly positive for CD133 and they also contained a large subpopulation of $NGFR^+$ cells (Figure 4.4C). However, other melanospheres, not expressing or expressing these markers at very low levels, exhibited similar or even stronger tumour initiating capacity *in vivo.* These findings may be compatible with the hypothesis that in melanoma different 'clones' of cancer stem cells may indeed exist.

The data presented in this thesis reflect the complex scenario of CSC markers and at the moment, no definitive consensus has been reached on the CSC phenotype for melanoma (Fang et al, 2005; Frank et al, 2005; Monzani et al, 2007; Keshet et al, 2008, Schatton et al, 2008; Quintana et al, 2008) and for other solid tumours as well (Dalerba et al, 2007; O'Brien, 2007; Becker et al., 2008; Ricci-Vitani et al, 2008; Shmelkov et al, 2008). Moreover, some recent

papers suggest that phenotypic heterogeneity may be also observed within adult stem cells of normal tissue such as bone marrow and intestine, and cells with different phenotype indeed display an equally regenerative ability (Stingl et al, 2006; Shackleton 2006, Alison et al, 2008; Sun et al, 2009, Cheng et al, 2009). To understand tumour heterogeneity, it has to be kept in mind that the parent-to-progeny relationship between CSCs and tumour bulk may not necessarily reflect the well conserved and predictable rules operating in normal tissue development. Conversely, tumour generation more likely represents an aberrant and highly unstable differentiation program due to the intrinsic genetic and epigenetic instability of cancer cellsThus tumour heterogeneity is likely to be the result of multifaceted mechanisms; among them variability of frequency, phenotype and even functional features of CSCs may be a relevant factor. Furthermore, the genetic and epigenetic instability that are characteristic features of tumour biology can induce cellular heterogeneity within the stem and the non stem cell population of the tumour, suggesting that cancer stem cells may be more dynamically regulated than hitherto suspected. Moreover new evidence proposes that cancer stem cell properties could be a functional state induced by environmental factors as recently shown for colon cancer and glioblastoma cancer stem cells (Vermeulen et al., 2010; Heddleston et al, 2009). Thus in line with this observation, the considerable phenotypic heterogeneity of melanospheres shown in this thesis might be a reflection of melanoma metastatic stem cell diversity that increases with genetic instability as tumours progress and

may indeed be the result of the emergence of distinct clones with melanoma initiating potential that better respond to different environmental signals.

The histological features of xenografts generated by melanospheres used in this study clearly resembled features of human melanomas thus recapitulating the original tumour heterogeneity. In particular, although melanospheres were found to be homogenously positive for Melan-A/Mart-1, this marker showed a heterogeneous expression in melanoma xenografts and in general the expression patterns of melanoma-associated markers and stem-related markers mirrored those of the patient's original tumour. In particular, very few cells could be found to be positively stained for CD133 and scattered cell nests positive for NGFR were detected in xenografts as well as in the original human tumours (Figure 4.9B). These patterns of staining were also found in a panel of primary and metastatic paired melanomas confirming that this heterogeneity is a rule in human melanomas. CD166, CD133 and Nestin have been reported to be expressed by a greater number of cells in human melanoma (Klein et al., 2007) as compared to nevi with no clear difference between primary and metastatic lesions although Nestin staining in early (I and II) stage melanomas has been shown to predict poor survival (Piras et al., 2010). Data reported in this thesis are in line with this observation with Nestin and CD166 are highly expressed in most of the cells composing the tumour mass (Table 4.8). Data in this thesis showed that in the analysed specimens, CD133 expression was detected both in primary and metastatic melanomas, with a similar expression pattern. Although $CD133^+$ cells are increased in melanomas compared to nevi (Klein et al., 2007; Sharma

et al., 2010), its prognostic significance and its association with progression in metastatic melanoma still remain to be established (Klein et al., 2007; Piras etal., 2010).

CD271 (NGFR) has been recently reported as a marker for the identification of melanoma cells strongly endowed with tumour initiating capacity and strong metastatic potential (Boiko et al., 2010). In the cases analyzed in this thesis, half of the metastatic lesions show an enhanced percentage of cells positive for NGFR with respect to the corresponding primary tumours (Table 4.8). Although NGFR seems to be crucial for the diagnosis of rare melanoma subtypes including desmoplastic melanoma (Lazova et al., 2010) and to specifically identify perineural invasion in malignant melanomas (MMs) (Chan and Tahan, 2010), the role of this receptor in melanomas is yet to be fully elucidated.

One additional important finding of this thesis is the clear observation that the ability to form tumours in immunocompromised mice is not a unique feature of melanospheres. In agreement with data from the literature (Rodolfo 1988), in this thesis it has also been shown that melanoma cells grown as adherent cells in medium containing FCS form tumours in SCID mice. However it is also nevertheless clearly demonstrated that the efficiency in tumour formation is higher for melanospheres as compared to adherent melanoma cells.

The *in vivo* experiments using immunocompromised mice, clearly suggest a higher enrichment in tumorigenic cells in melanospheres with respect to adherent cells. Indeed, tumour take and latency of xenografts generated in primary recipients by melanospheres and adherent melanoma cells are different with a

high statistical significance (P<0.0001) as evaluated by an accurate multiple regression model. Moreover, as far as the xenografts serially transplanted into subsequent hosts are considered, the difference in tumour take between the two groups emerges even more clearly, with efficiency in tumour formation for melanospheres being 100% while being 52% for adherent cells (Table 4.5). These data, together with the evidence that melanospheres could have been generated from their corresponding adherent melanoma cells, strongly suggest that putative melanoma stem cells are likely contained both among melanospheres and adherent melanoma populations. Nevertheless, because of the absence of molecular marker(s) or lineage tracing *in vivo,* our data did not definitively address the issue of the relative frequency of CSCs in melanosphere and adherent melanoma cells.

In agreement with these quantitative differences, xenografts derived from adherent melanoma cells qualitatively diverged from xenografts generated by melanospheres. In general, adherent cell-derived tumours displayed a highly differentiated phenotype, with a uniform expression of Melan-A/Mart-1 and an enhanced level of MITF expression, which correlates with a decrease invasive potential of melanoma cells (Carreira et al., 2008) (Fgiure 4.10A). Ultrastructural analysis through electron microscopy revealed that tumours generated from adherent cells do contain highly differentiated melanoma cells, enriched in mature melanomsomes in comparison to melanosphere-derived xenograft tumours that are characterized by the presence of more immature melanosomes; moreover adherent cells are enriched in intra cellular vacuoles, characteristic of

cells possibly undergoing to autophagic processes that are almost absent in sphere-derived tumours (Figure 4.10C).

This scenario could indeed be also compatible with the hypothesis that melanoma adherent cells are mainly finally differentiated cells that, while still maintaining proliferation potential, may form tumours *in vivo*, but these tumours display strong features of differentiation and are endowed with very limited transplantability capacity in subsequent animal hosts.

In conclusion, the present data provide further evidence that human melanoma is a heterogeneous cancer, composed by cells with different characteristics. We further show that melanoma cells selected by their ability to form melanosphere cells in stem cell culture medium retain the ability to efficiently regenerate human melanoma in a mouse model, which is the crucial characteristic that functionally defines CSC.

Moreover, results presented in this thesis add to the existing evidence that human melanoma is a heterogeneous cancer composed of cells with different characteristics and suggest that melanospheres and their corresponding tumours in immunocompromised mice represent a reliable model to analyze melanoma biology. A better understanding of the fate of melanosphere-derived cells inoculated *in vivo*, and their response to stimuli derived from the tumour stroma, will offer the possibility to better understand the mechanisms driving melanoma formation and progression. This new knowledge will be instrumental in designing novel, more efficient therapies targeting CSCs in human melanoma.

CHAPTER 6: MELANOMA STEM CELLS AND THEIR CROSS-TALK WITH THE IMMUNE SYSTEM

6.1 Introduction

The previous parts of this thesis were focused on the identification and *in vitro* isolation of melanoma cells with stemness properties and strong tumour initiation capacity *in vivo*. Studies reported in this chapter were aimed at investigating the immunological properties of melanoma stem cells.

According to the CSC theory, tumour microenvironment acquires novel significance and it is likely to provide the "niche" influencing CSCs fate. Thus, considering that melanoma is an immunogenic tumour, with the final aim of designing immune–based intervention targeting CSCs, it was of particular interest to define the reciprocal interaction of melanoma initiating cells with the immune system.

It is well known that malignant melanomas produce and express receptors for different cytokines, chemokines and growth factors which, through autocrine and paracrine mechanisms of action, enable melanoma cells to growth autonomously and to acquire metastatic phenotype. Autocrine production of IL-8 and sometimes IL-6 and IL-10 stimulated melanoma proliferation, while growth

factors such as IL-1 and TGF-β1 either produced by melanoma cells or by stromal cells, modulate tumour microenvironment to promote tumour growth and invasion (Ikushima and Miyazono, 2010). Furthermore, many chemokines such as CXCL1-2, CXCL5-8, CCL2, CCL5, CCL21 and CCL27 are known to sustain melanoma growth and progression (Richmond et al., 2009) and other chemokines (CCL2-4, CXCL9, CXCL10) are associated with the recruitment of cells of the immune system, such as pro-tumorigenic macrophages (Harlin et al., 2009).

From an immunological point of view, evidence has been provided for a favourable prognostic role of the immune system in cancer patients. However, despite significant progresses in tumour immunology, current immuno-based therapies targeting antigens highly expressed in cancer cells failed to induce clinically effective anti-cancer responses. This breakdown can be due to the immune-suppressive mechanisms active in cancer patients; this suppressive environment selectively might protect CSCs from immune effectors cells. Indeed, induction of tolerance is a key feature of normal mesenchymal stem cells that display immunosuppressive properties (Spaggiari et al., 2009; Di Ianni et al., 2008). Moreover, neural stem cells, through the release of soluble mediators, exert immunomodulatory effect on peripheral T cells by suppressing their activation and proliferation (Kim et al., 2009). These 'physiological' mechanisms parallel mechanisms of immune evasion relevant to neoplastic development and progression (Le Blanc and Ringden, 2007). In a tumour setting, glioblastoma multiform CSCs show low immunogenicity and high suppressive activity

suggesting that the low differentiation stage of CSCs could be associated with a weaker expression of molecules that are crucial for T cells and NK recognition (Di Tomaso et al., 2010).

Secretion activity of stem cells has been proved to be extensive and to play a relevant role in regulating proliferation and differentiation processes. In particular, the autocrine production of IL-4 plays an important role in increasing self-renewal and survival of colon CSCs (Todaro et al., 2007; IL-6 is involved in Notch-mediated signalling that is crucial for breast SC maintenance (Sansone et al., 2007) and is known to contribute to haematopoietic stem cell maintenance (Kang et al., 2007).

Based on these evidences, this part of the thesis explored the immune-related phenotype of melanoma initiating cells with the aim of assessing their possible interaction with the immune system in term of susceptibility to recognition and/or ability to negatively modulate the anti-tumour response, as recently suggested for $ABCB5^+$ melanoma stem cells (Schatton et al., 2010). The expression of class I and II HLA (Human Leukocytesa Antigen) as well as that of the immune regulatory molecules CTLA-4 (Cytotoxic T-Lymphocytes Antigen-4), PD-1 (Programmed Death-1) and their ligands was analysed in melanospheres and in their corresponding adherent melanoma cells. Moreover, melanospheres were also assessed for their secretion profile both at mRNA and protein level.

6.2 Results

6.2.1 Expression of cell surface immune-related molecules by melanospheres

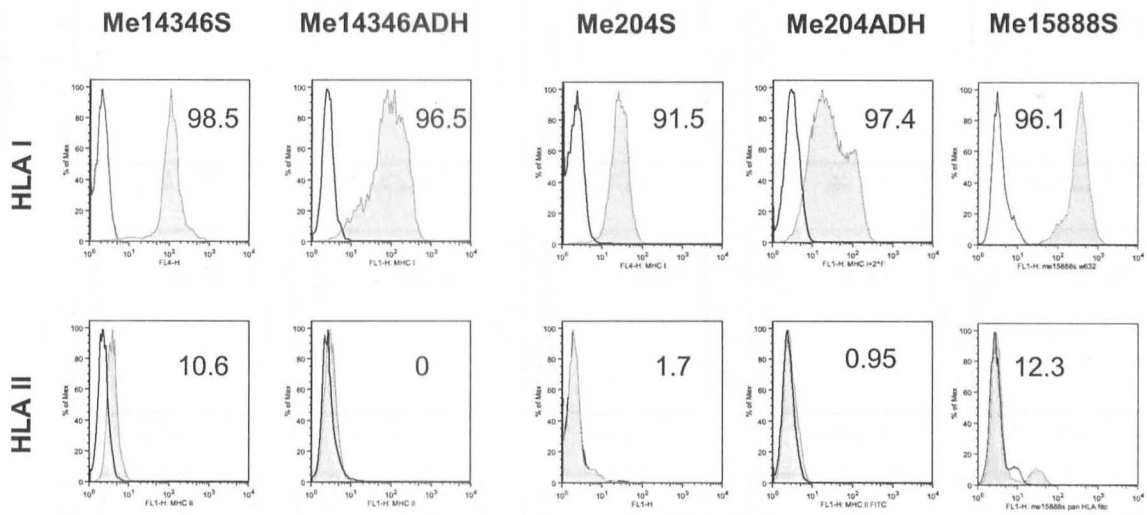
Phenotypical analysis by flow cytometry revealed that HLA class I (are expressed by all the melanospheres, while only a limited expression for HLA-class II (Major Histocompatibility Class II, MHC II) could be detected in Me14346S and Me15888S. Of note, although all the cells within melanospheres and adherent cells express HLA class I (Figure 6.1A), the fluorescence intensity is slightly lower on melanospheres, suggesting that these cells express lower level of HLA class I in comparison to their corresponding adherent cells, confirming in melanoma data previously reported for glioblastoma SCs (Di Tomaso et al., 2010).

The expression of molecules involved in the modulation of the activation status of T cells was also examined. Results reported in figure 6.1B showed that PD-1 and its ligand PD-L1 are expressed by the samples tested with no major differences between melanospheres and adherent cells. Conversely, the expression of CTLA- 4, an additional inhibitory molecule, is expressed at higher level in melanospheres as in comparison to adherent cells. Notably this difference is even more pronounced when the expression of CTLA-4 is examined without previous cell permeabilization indicating that CTLA-4 molecules are exposed directly on the cell surface and available for functional triggering. Although at low level, the CTLA-4 ligand CD86 is detectable on all the analysed

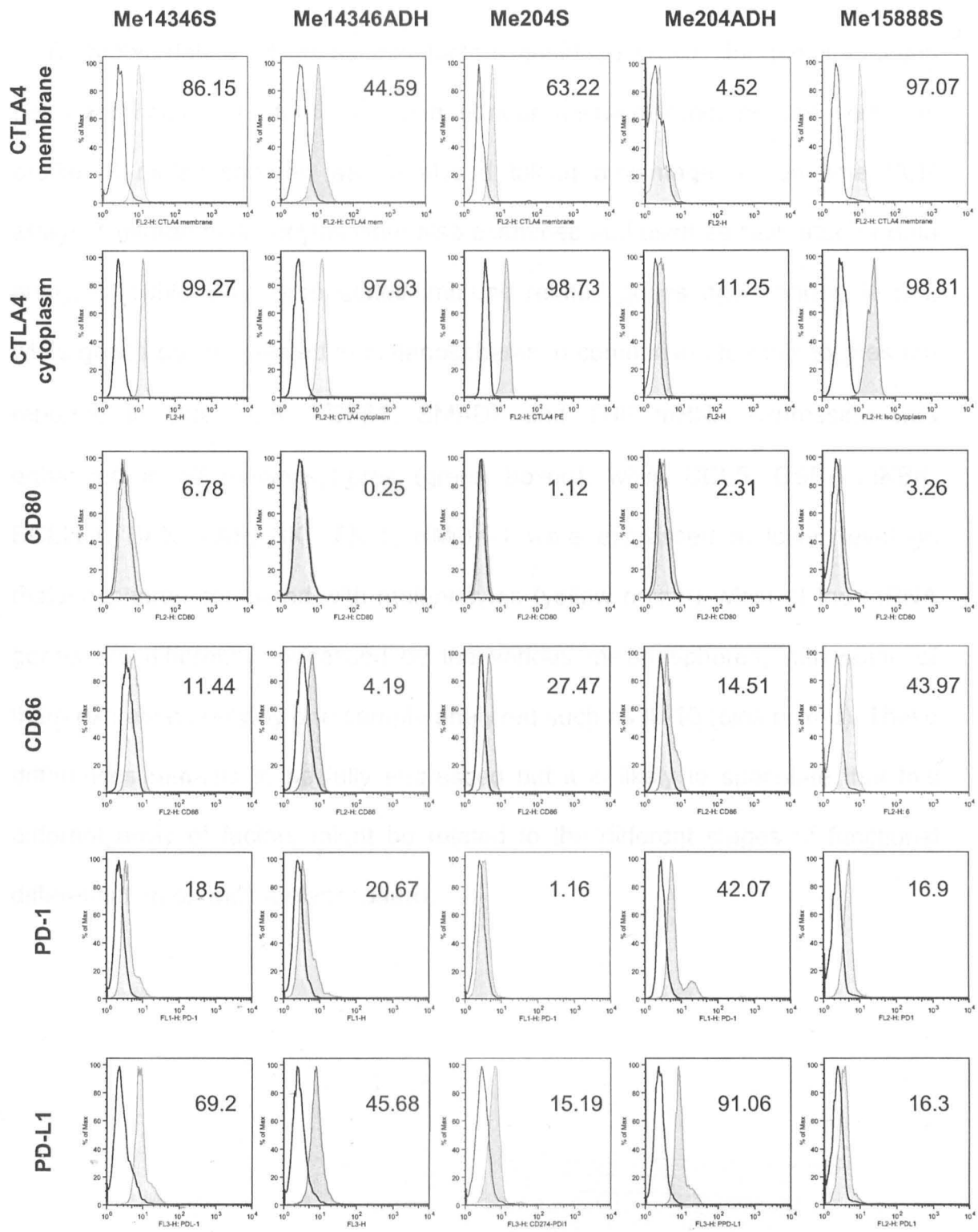
samples suggesting the possibility of autocrine activation. No CD80 expression was evidenced.

Figure 6.1. Expression of immune related molecules on melanospheres and adherent cells. Black line represents Isotype controls; gray filled histograms represent the specific antibody. Numbers indicate the percentage of positive cells.

Panel A



Panel B


Me14346S
Me14346ADH
Me204S
Me204ADH
Me15888S
CTLA4 membrane
86.15
44.59
63.22
4.52
97.07
CTLA4 cytoplasm
99.27
97.93
98.73
11.25
98.81
CD80
6.78
0.25
1.12
2.31
3.26
CD86
11.44
4.19
27.47
14.51
43.97
PD-1
18.5
20.67
1.16
42.07
16.9
PD-L1
69.2
45.68
15.19
91.06
16.3

6.2.2 Expression of immune-related genes

To better define which soluble factors could be crucial for the interaction between melanoma stem cells and tumour microenvironment, the secretion profile of melanospheres was analysed taking advantage of real-time PCR arrays. Cultured melanocytes were also examined and used as calibrator for data analysis (Table 6.1). Upregulated immune related genes are reported in red, while genes downregulated in melanospheres in comparison to melanocytes are reported in blue. IL-8, TGFβ1, SMAD7 and TNF mRNA expression was enhanced in all melanospheres (green boxed), while CCL5, CSF-1, IKBK, BCL2L1, BAX, FAS, SKI, FN-1, HMOX-1 were expressed at lower level on melanospheres compared with melanocytes (yellow boxed). Most of the mRNA genes are differently expressed by the various melanospheres, with some of them expressed only by one sample analyzed such as IL-10 (pink boxed). These differences remains to be fully addressed but it is likely to speculate that this different array of factors might be related to the different stages of functional differentiation of each melanosphere.

Table 6.1. Expression of immune-related genes on melanospheres. Melanocytes are used as calibrator.

	[2]RQ				
Gene	**Me14346S**	**Me204S**	**MeJR8S**	**Me15888S**	**[1]Melanocytes**
IL-1β	-[3]	-	-	77.29	-
IL-6	-	-	-	-	1
IL-7	1.39	1.37	0.07	-	1
IL-8	7.49	9.01	3.66	11.62	1
IL-10	-	-	-	382.9	-
IL-12a	-	135.26	6.64	-	-
IL-18	-	-	-	132.18	-
CCL2	0.33	-	0.02	1.81	1
CCL3	-	122.33	12.89	-	-
CCL5	0.73	0.23	0.07	0.55	1
CCR4	-	-	-	-	-
CXCL10	-	-	-	0.21	1
CXCL11	-	-	-	0.85	1
CSF1	0.19	0.14	0.02	0.21	1
CTLA-4	-	0.18	-	11.72	1
CYP7A-1	-	-	-	-	-
AGTR2	-	-	-	-	-
STAT3	0.17	1.22	0.19	0.50	1
NFKB	0.43	4.59	1.03	0.78	1
IKBK	0.54	0.30	0.16	0.59	1
TGFβ1	11.95	45.59	17.84	10.68	1
CD3E	-	-	10.82	-	-
CD4	1.31	-	-	0.50	1
CD40	0.03	11.98	1.06	-	1
CD38	-	-	-	-	1
CD68	0.56	7.19	3.50	1	1
CD80	132.02	-	-	-	-
CD86	-	-	-	-	1

	[2]RQ				
Gene	Me14346S	Me204S	MeJR8S	Me15888S	[1]Melanocytes
HLADR A	-	0.04	0	0	1
HLADR B	-	-	-	-	1
PTGS2	0.06	0.11	0.13	-	1
COL45A	-	26.96	3.29	-	1
VEGF	0.60	1.81	0.55	0.90	1
C3	0.02	2.31	2.72	-	1
ACE	-	-	-	-	1
ICOS	-	-	-	7.37	1
NOS2a	4.15	-	-	29.5	-
BCL2	1.68	0.36	0.16	1.06	1
BCL2L1	0.26	0.69	0.14	0.67	1
BAX	0.48	0.54	0.12	0.69	1
FAS	0.08	0.05	0.01	0.64	1
SMAD3	1.91	2.55	0.31	1.98	1
SMAD7	1.79	18.99	3.97	2.69	1
SKI	0.58	2.24	0.48	0.63	1
FN-1	0.26	0.52	0.10	0.94	1
TNF	62.42	42.66	20.29	251.77	-
ICAM-1	0.70	13.35	2.29	1.73	1
HMOX-1	0.26	0.65	0.11	0.38	1
LRP-2	-	-	-	3694.87	-
ECE1	1.07	6.08	1.58	0.84	1

[1]: calibrator; [2]: RQ values calculated using the equation RQ=2-ΔΔ C_t. ΔΔC_t values were calculated by subtracting the ΔC_t of the calibrator from the ΔC_t value of each target; [3]: Absent genes defined as genes with ΔC_t above 25 cycles. 0 = RQ< 1E-3.

6.2.3 Secretion profile of melanoma stem cells

After this initial evaluation, quantitative analysis of cytokines and chemokines released by melanospheres in culture supernatants was performed by flow cytometry through Cytokine Beads Array (CBA). The secretion profile of melanospheres was compared to that of adherent melanoma cells and melanocytes. The secretion level of each cytokine and chemokine is reported in Table 6.2.

Data confirmed at the protein level the profile obtained with mRNA analysis; all the melanospheres produced IL-8 and CCL-2 while IL-10 is exclusively released by Me15888S. Conversely, IL-6 and IL-4 were not detected in any of the melanosphere-conditioned supernatant.

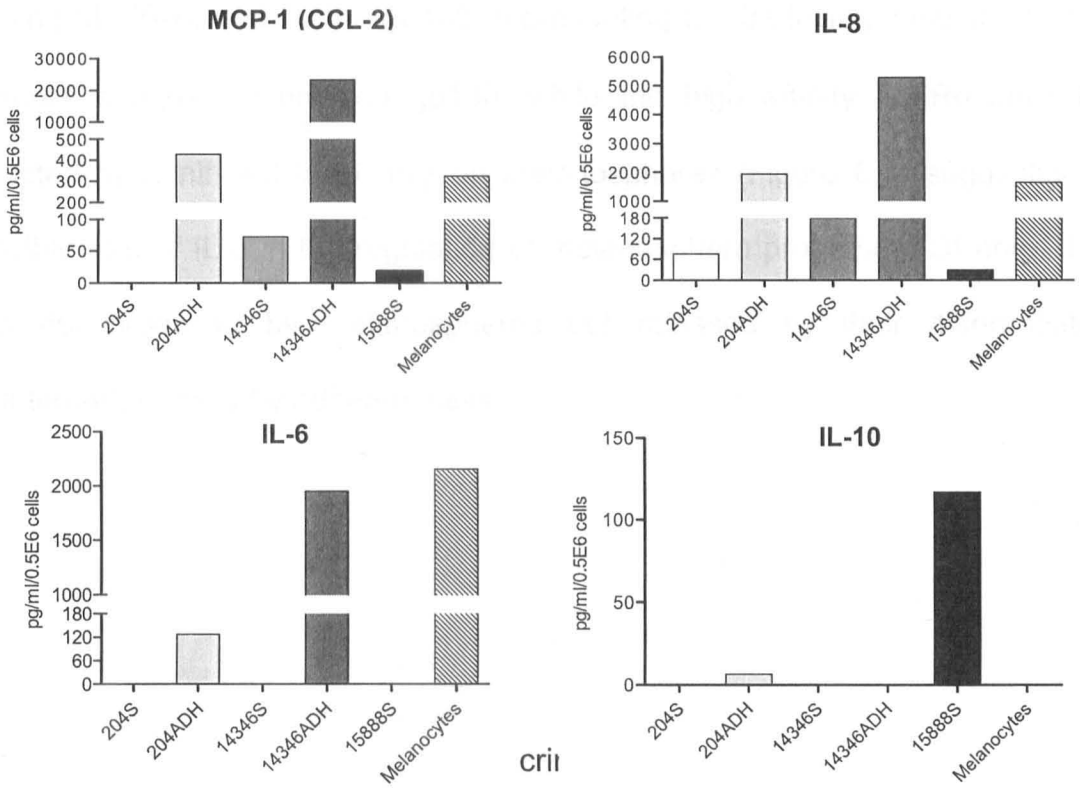
Table 6.2. Cytokines and chemokines secretion level.

		[pg/ml][1]					
		Me14346		**Me204**		**Me15888**	**Melanocytes**
		ADH	*S*	*ADH*	*S*	*S*	*ADH*
Cytokines	**IL-1β**	11.5	[2]-	1.85	-	-	-
	IL-2	-	-	-	-	-	-
	IL-4	-	-	-	-	-	-
	IL-5	-	-	-	-	-	-
	IL-6	1950.59	-	[3]127.56	-		430.66
	IL-8	1450.22	231.220	2119.73	150.02	20.11	2515.00
	IL-10	-	-	6.45	-	116.92	-
	IL-12p70	-	-	-	-	-	-
	TNFα	4.01	-	1.53	-	-	-
	TNFβ	-	-	-	-	-	-
	INFγ	-	-	-	-	-	-
Chemokines	**G-CSF**	-	-	-	-	-	-
	MCP-1 (CCL2)	23262.63[3]	72.41	428.13	-	17.97	323.48
	MIP1α	102.97	-	17.91	-	-	-
	MIP1β	12.58	-	-	-	-	-
	MIG	412.71	-	-	-	-	-

[1]: pg/ml: pg of released molecule for ml of culture supernatants normalized for 0.5E6 cells. Values reported are mean of two replicates obtain after subtraction of the values detected in the unconditioned culture medium. [2]: undetected. [3]: out of range of calibrator curve.

In this analysis, melanocyte and adherent cell-conditioned media were also considered. Interestingly, in comparison to melanospheres, these cells product IL-8 and CCL-2 at higher level. The same trend was detected for IL-6. This cytokine was produced by melanoma adherent cells and melanocytes while it was undetectable in melanosphere-conditioned supernatants (Table 6.2 and Figure 6.2). These data are in line with published findings reporting that advanced melanomas produce high level of IL-6 known to exert both autocrine and paracrine roles in melanoma biology (Lazar-Molnar et al., 2000).

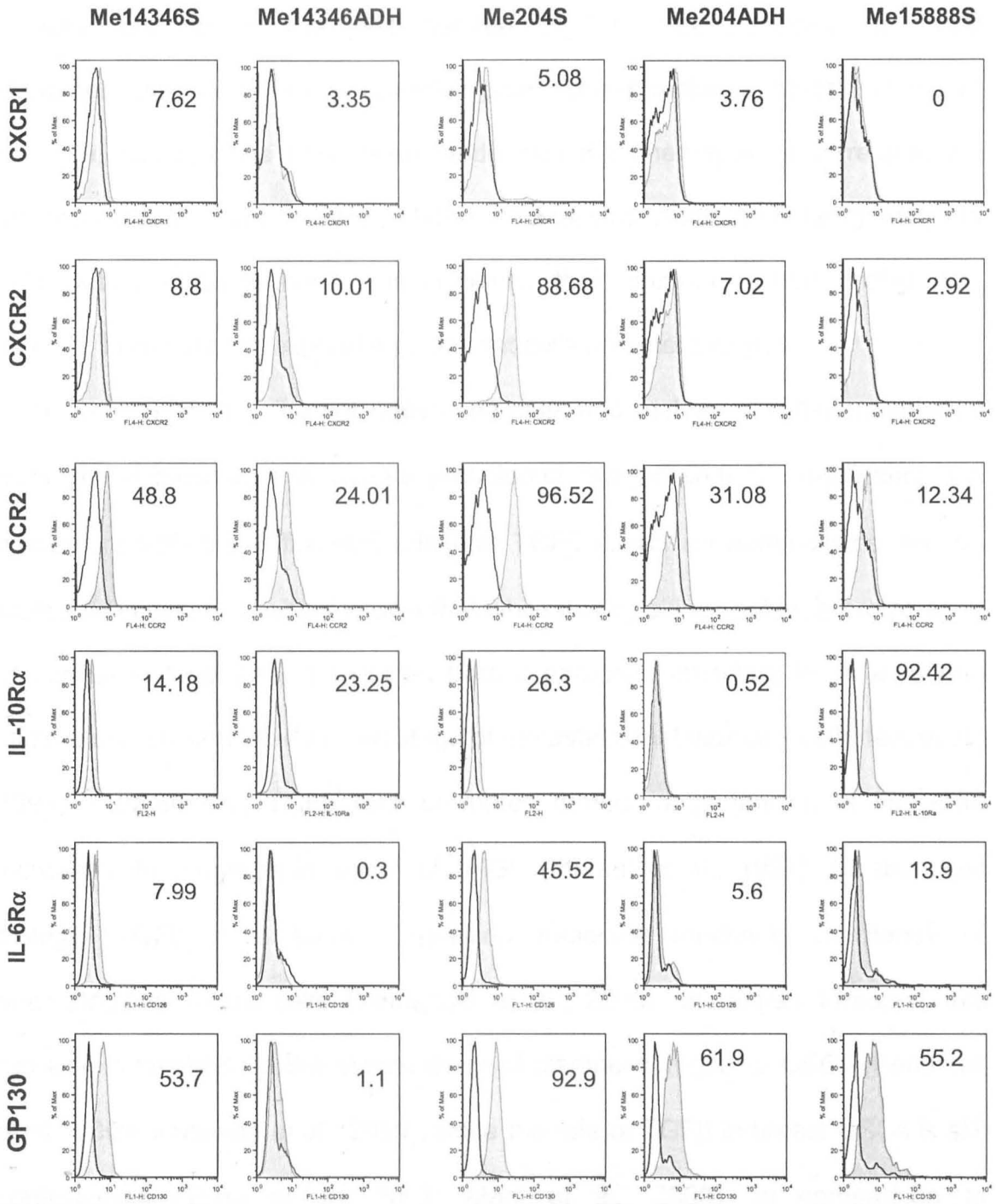
Figure 6.2. Cytokines and Chemokines secretion levels. Melanospheres produce lower level of IL-6 and CCL-2 with respect to adherent melanoma cells and melanocytes. IL-6 is exclusively produced from melanoma adherent cells and IL-10 is only produced by Me15888S. On the X axes are reported name of the analyzed samples; on the Y axes are reported pg/ml of released cytokines normalized for 0.5E6 cells after subtraction of the values detected in the unconditioned culture medium.



criı of receptors, specific for the cytokines/chemokines released by melanospheres or by more differentiated melanoma cells, here represented by melanoma cells growth as adherent monolayer, was also examined. Data are reported in Figure 6.4. The high affinity IL-8 receptor CXCR2 was expressed in all the analyzed samples with the exception of Me204ADH, while only a fraction of Me14346S and Me204S was positive for CXCR1. The CCL-2 receptor (CCR-2) is expressed both on melanospheres and adherent cells with an enhanced expression by Me204S and Me14346 S in comparison to adherent cells.

Me15888S, that produced IL-10, expressed enhanced level of IL10Rα thus suggesting a possible autocrine role of IL-10 in influencing melanospheres biology. IL-6 receptor is composed by the α subunit, responsible for IL-6 specific binding (IL-6Rα), and by the gp130, representing the traducing subunit. All the samples analysed expressed gp130, while the high affinity IL-6Rα could be detected at significant level only on melanospheres (Figure 6.3) suggesting a possible role of IL-6 in the regulation of melanosphere properties. Of note, IL-6 was not produced by melanospheres but released by their differentiated counterpart, namely by adherent cells.

Figure 6.3. Expression of Cytokines and Chemokines receptor. Black lines represent Isotype controls; gray filled histograms represent the specific antibodies. % of positive cells is reported in each histogram.



6.3 Discussion

Data reported in this last session of the thesis provide an initial characterization of the immune-related phenotype of melanospheres. Whenever possible, comparisons have been made with the phenotype of corresponding adherent cells and with *in vitro* cultured melanocytes. Although heterogeneity did exist within melanospheres, nevertheless their immune-related profile was different from their corresponding adherent cells and melanocytes.

In comparison to adherent melanoma cells and melanocyte, melanospheres displayed significantly enhanced expression of TGFβ (Tab 6.1). This finding is in agreement with the differential role that TGFβ exerts on melanocytes and on melanoma cells. It is in fact known that while this cytokine limits the proliferation of normal melanocytes, it switches from a paracrine inhibitory to an autocrine stimulatory function in advanced stage of metastatic melanomas (Vetterlein et al., 1995). Furthermore, TGFβ also promotes tumour angiogenesis *in vivo* and increases the angiogenic action of bFGF (Moretti et al., 1997). In stem cell biology, TGFβ is a known signalling molecule mediating dormancy of haematopoietic stem cells (Yamazaki et al., 2009). In human tumours, this cytokine is involved in the maintenance of stemness in glioma CSCs (Penuelas et al., 2009; Ikushima et al., 2009), while the role of TGFβ in breast CSCs is still controversial (Tang et al., 2007; Mani et al., 2008). In comparison to melanocytes, melanospheres displayed also enhanced expression of SMAD7 transcript as compared to melanocytes, in accordance to the observation that

SMAD7 regulates neural stem/progenitor cell proliferation in a TGFβ-independent manner (Krampert et al., 2010).

In the 6.2.3 section, data obtained by mRNA expression analysis have been confirmed at protein level and assessment of the quantity of cytokines/chemokines in the melanoma-conditioned media was obtained taking advantage of a flow cytometry based assay, known as CBA. Interestingly melanospheres displayed a secretion profile distinct from that of the adherent melanoma cells and melanocytes. Differences were evident for those cytokines whose role in melanoma progression has been previously reported (Lazar-Molnar et al., 2000). In particular, IL-8 and CCL-2 (also known as macrophage chemoatactic protein 1 MCP-1), were produced at considerably lower level by melanospheres than adherent cells (Table 6.2 and Figure 6.2).

The differential expression of CCL-2 between melanospheres, displaying a more aggressive behaviour *in vivo* (see chapter 4), and adherent cells, is in line with the observation that higher amount of CCL2 is secreted by melanoma cell lines with lower metastatic and invasive potential. These *in vitro* data mirror the *in vivo* situation in which expression of CCL-2 mRNA is higher in melanoma in situ and decreases in more invasive tumours (Varney et al., 2005). Moreover, it has been observed that CCL-2 in melanoma exerts different functions depending on its secretion level and it works as chemo-attractant for pro-tumorigenic macrophages when secreted at low level by tumour cells. Low level of CCL-2 production by melanospheres may thus be associated with the capacity of

melanoma initiating cells to generate a tumour promoting microenvironment (Nesbit et al., 2001).

IL-8 exerts an autocrine role in sustaining melanoma proliferation, through interaction with its receptors (CXCR1 and CXCR2), that are expressed at high level on melanoma cells and melanocytes (Varney et al., 2003). Recent evidences also showed that IL-8 receptors are upregulated under hypoxic conditions and have a role in tumour angiogenesis. CXCR2 is also involved in melanoma cell migration and invasion (Gabellini et al., 2009) and data of this thesis showed that CXCR2 was expressed on all the analysed melanospheres while CXCR1 was present on a subpopulation of cells within melanospheres, with the exception of Me15888S (Figure 6.3). A role of IL-8 and CCL-2 in modulating normal or cancer stem cell biology has not been yet addressed although IL-8 receptor CXCR1 has been proposed as marker for the definition of the breast cancer stem cells subpopulation within the $ALDH^+$ cells (Ginestier et al., 2010).

IL-6 could not be detected in melanosphere supernatants while it was highly produced by adherent cells and cultured melanocytes. A function of IL-6 and IL-10 in influencing stemness properties has been clearly demonstrated in the literature. Exogenous added IL-6 was shown to induce neural and glial differentiation of murine neural stem cells (Islam et al., 2008) and it has been recently demonstrated that IL-6, secreted by differentiated astrocytes, induced specific neuronal differentiation of rat neural hyppocampal progenitor cells (Oh et al., 2010). Analogously, IL-6 produced by human microglia negatively influenced

neurosphere self-renewal (Balasubramaniam et al., 2009). As concern melanomas, the role of IL-6 is once more controversial when early or late stage melanomas are considered. In particular, the growth of early stage melanomas are inhibited by IL-6 released by fibroblasts, conversely, metastatically competent, advanced stage melanomas are resistant to this anti-proliferative function (Sun et al., 1992; Silvani et al., 1995). Furthermore, IL-6 sustains proliferation of differentiated melanoma while is not necessary for early melanoma replication (Lu et al., 1992). These recent evidences favour the hypothesis that IL-6 may have a role in supporting differentiated cells and being melanospheres likely composed by undifferentiated melanoma cells they did not require this cytokine for their survival. Nevertheless data of this thesis indicated that melanospheres express IL-6 receptor but the biological significance of this expression remains to be addressed. IL-6, interacting with Notch signalling, affects self-renewal in beast CSCs (Sansone et al., 2007) and thus, also in melanoma model, IL-6 produced by the differentiated melanoma cells or by tumour stroma may act in a paracrine way and influence stemness properties of melanoma stem cells in accordance to the recent discussed hypothesis that cancer stem cells could be dynamically regulated by tumour microenvironment (Heddelston et al.; 2009).

IL-10 was detected in sera of patient with advance stage malignant melanoma. IL-10 is a well known immunosuppressive cytokine that affects the expression of HLA class I on different cells type including antigen presenting cells. Most of the melanoma cell lines express IL-10 receptor and its expression is associated with

a pronounced down-regulation of the HLA class I molecule (Yue et al., 1997) and of MHC class I chain-related proteins A (MICA). These IL-10 mediated effects directly protect melanoma cells from recognition by both T and NK cells.

The involvement of IL-10 in stimulating self-renewal of haematopoietic stem cells has been recently suggested (Kang et al., 2007). Furthermore, glioma stem cells expressed enhanced level of IL-10 mRNA in comparison to the correspondent adherent glioma cell lines, with a correlation between the level of IL-10 mRNA expression and the pathological grade of gliomas (Qiu et al., 2010). Adipose derived stem cells (ASCs) isolated from breast cancer patients display significantly higher level of IL-10 and TGFβ mRNA than ASCs isolated form normal individuals and the culture supernatant of ASCs induces *in vitro* regulatory T cells (Razmkhah et al., 2010).

Results presented in sections 6.2.3 show that IL-10Rα was expressed both on melanospheres and adherent cells, and was strongly upregulated on Me15888S that uniquely produced significant amount of IL-10. This phenotype suggests a possible autocrine role of IL-10 in Me15888S maintenance and also a possible a paracrine role of this cytokine in influencing melanoma behaviour.

Immunotherapy has a long history with striking but limited success in patient with melanoma. The limitations of melanoma immunotherapy stem from tumour-induced mechanisms of immune evasion that cause the host to be tolerant to tumour antigens. For instance, melanoma inhibits the maturation of antigen-presenting cells, thus preventing full T-cell activation and diminishing the effector antitumour immune response. New immunotherapy, targeting critically regulatory

elements of the immune system, may overcome tolerance, leading to a more effective antitumour response. These include monoclonal antibody that blocks CTLA-4 thus preventing inhibitory signals that down-regulate T-cell activation. Moreover, since the T-cell proliferation and cytokine production can be inhibited also by the interaction of PD-1 with its ligand PD-L1 and by the interaction of PD-L1 with CD80, the simultaneous blockade of CTLA-4 and PD-1 has been shown to be more than twice effective in promoting the rejection of melanoma in *in vivo* murine models (Curran et al., 2010). Interestingly, CTLA-4 and also the co-stimulatory molecules PD-1, PD-L1 and CD86 are expressed by the melanospheres and adherent cells here analyzed, with membrane CTLA4 and CD86 prevalently expressed on melanospheres (Figure 6.1A and 6.1B). These observations are in line with the phenotype reported for ABCB5+ melanoma initiating cells endowed with stronger ability to inhibit peripheral blood mononuclear cell (PBMC) proliferation and enhancing T regulatory cells induction via CD86 (Schatton et al., 2010). Analogous immunosuppressive properties are displayed by mesenchymal stem cells (Di Ianni et al., 2008) and by gliomacancer initiating cells that, inhibit T-cell proliferation and activation thorough PD-L1. Interestingly, exposure of glioma initiating cells to differentiation stimuli causes the lost of their immunosuppressive properties (Wei et al., 2010).

Taken together the data presented in this part of the thesis underline a strong relation between the immune system and the putative melanoma stem cells. This relationship indicates that CSCs are indeed well equipped to evade immune-recognition and able to exploit immune-related factors for improving their stem

cell-related functions. Thus, immunotherapy approaches should consider strategies targeting CSC subpopulation. In addition, it will be mandatory to combine novel treatments efficient against CSCs with the most recent therapies targeting the immunoregulatory molecules CTL-4, and PD-1 although the precise role of these two molecules and that of CD86 and PD-L1 expressed on CSCs remains to be identified.

CHAPTER 7: FUTURE PERSPECTIVES

Future experiments will be aimed at defining the reciprocal interaction between melanoma initiating cells and the tumour microenvironment, stroma and the immune system, with particular attention to the ability of soluble factors in regulating melanoma heterogeneity, plasticity and tumour maintenance. To this aim, the following lines of research will be pursued:

1. To elucidate the functional role of the identified chemokines/cytokines in affecting self-renewal and differentiation of melanoma stem cells. Experiments will be performed *in vitro* and *in vivo* taking advantage of neutralizing antibodies directed against each cytokine and using agonist/antagonist antibodies directed to the cytokine receptors of interest. Experiments with siRNA will be also designed.

2. Considering that melanospheres display a heterogeneous phenotype, the possibility to identify different subpopulations of cells within melanospheres will be explored. Starting from the observation that as in breast mammospheres (Pece et al., 2010,) also melanospheres contain a slow

cycling fraction of cells (Roesch et al., 2010), the differential response of these two populations to modulating cytokines/chemokines will be carefully explored.

3. Interaction with stromal cells is crucially important for tumour development. The influence of keratinocytes or fibroblast, in modulating the properties of the different subpopulation of melanospheres will be investigated.

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viability and maintenance. Proc Natl Acad Sci U S A. 2007 Oct 9;104(41):16158-63.

LIST OF ABBREVIATION

ABC	ATP-binding cassette
ACE	Angiotensin Converting Enzyme
ADAM	A Disintegrin And Metalloprotease
ADH	Adherent cells
AGTR2	angiotensin II type 2 receptor
AJCC	American Joint Committee on Cancer
AKT	V-akt murine thymoma viral oncogene homolog
ALCAM	Activated Leukocytes Cell Adhesion Molecule
ALDH	Aldehyde DeHydrogenase
ALM	Acral Lentiginous Melanoma
AML	Acute Myeloid Leukaemia
APAF-1	Apoptotic Protease Activating Factor-1
APC	Adenomatous Polyposis Coli
APC	AlloPhycoCyanin
ARF	Alternatite Reading Frame
ASCs	Adipose derived Stem Cells
BAX	Bcl-2-Associated X Protein
BCA	Bicinchoninic Acid
BCL2	B Cell Lymphoma gene-2
BCL2L1	BCL2-like 1
BCL9	B-Cell Lymphoma 9
BCNU	Carmustine
bFGF	Basic Fibroblast Growth Factor
Bmi1	Bone morphogenic insertion 1
BMP	Bone Morphogenic Protein
BRAF	V-raf murine sarcoma viral oncogene homolb B1
BRN-2	POU domain class 3 transcriptor factor-2
BSA	Bovine Serum Albumin
C3	Complement component C3
CBA	Cytokine Beads Array
CCL	C-C chemokines ligand-
CCP	CisPlatinum
CCR2	C-C chemokine receptor type 2
CD	Cluster Differentiation
CDH	Cadherin
CDK-	Cyclin-dDependent Kinase
CDKN2A	Cyclin-Dependent Kinase inhibitor 2A
cDNA	complementary DNA
CK1a	Casein Kinase 1a
CML	Chronic Myeloid Leukaemia
CoA	CoActivator
COL45A	Collagen 45A gene

CoR	CoReceptor
CPB	CREB Binding Protein
CREB1	CAMP responsive element binding protein 1
CSCs	Cancer Stem Cells
CSF-1	Colony-Stimulating Factor 1
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
CXCL-	C-X-C chemokines Ligand -
CXCR-	C-X-C chemokine Receptor type-
CYP7A-1	Cytochrome P450, family 7, subfamily A, polypeptide 1
DELL	DElta-Like Ligand
DM	Desmoplastic Melanoma
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNER	Delta/Notch-like EGF-Related receptors
dNTP	nucleotides
DTIC	Dacarbazine
DTT	DiThioThreitol
Dvl	Disheveled
ECE1	Endothelin-Converting Enzyme-1
ECM	ExtraCellular Matrix
Edn3/ET	Endothelin 3
EdnrB	Endothelin 3 receptor B
EDTA	EthyleneDiamineTetraacetic Acid
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular signal-Regulated Kinase
ESA	Epithelial Specific Antigen
FABP-4	Fatty Acid Binding Protein 4
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FAS	Fatty Acid Synthase
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FITC	Fluorescein IsoThioCyanate
FN-1	FibroNectin-1
Fu	Fused
Fz	Frizzled
GC	Growth Curve
GF	Growth Factor
GMB	GlioBlastoma Multiform
GSIs	γ-Secretease Inhibitors
GSK3β	Glycogen Synthase Kinase 3β
HD	Homozygous Deletion
HDAC	Hystone DeACetylase

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
Hh	Hedgehog
HIF	Hypoxia-inducible factor
HLA	Human Leukocytes Antigen
HMOX-1	heme oxygenase (decycling) 1
HRP	HorseRAdish Peroxidase
HSCs	Haematopoietic Stem Cells
i.p.	Intra περιτονεουμ
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICOS	anti-Inducible T-cell Co-Stimulator
IGF	Insulin-like Growth Factor
IKBK	nuclear factor NF-kappa-B inhibitor kinase beta
IL-	InterLeukin -
INF	Interferon
Jag	Jagged
JARID1B	jumonji/ARID1 (JARID1) histone 3 K4 (H3K4) demethylases
kit	c-kit tyrosine kinase receptor
LDH	Lactate dehydrogenase
LEF	Lymphoid-Enhancing Factor
LMM	Lentigo Maligna Melnaoma
LRP-2	Low density lipoprotein-related protein 2
M	melanosomes
MAGE	Melanoma antigen family
MALM-1	MAstermind-Like protein 1
MAPK	Mitogen Activated Protein Kinase
MART-1/Melan A	Melanoma Antigen Recognized by T cells-1/Melanoma Antigen A
MATP	Membrane-Associated Transporter Protein
MB	MedulloBlastoma
MC1R	MelanoCortin 1 Receptor
MCAM	Melanoma Cell Adhesion Molecule
MCP-1	Monocyte Chemoattractant Protein 1
MDM2	Murine Ddouble Minute 2
MDR	MultiDrug Resistance
MDR1	MultiDrug Resistance Protein-1
MEK	MAPK/ERK Kinase
MGMT	O-6-methylguanine-DNA methyltransferase
MHC	Major Histocompatibility Complex
MIG	Monokine Induced by Gamma-Interferon
MIP1	Macrophage Inflammatory Proteins-1
MITC	5-(3-methyltriazen-1-yl)imidazole-4carboximide
MITF	Microphtalmia-associated Transcription Factor
MM	Myxoid Melanoma
MMs	Malingnat Melanomas
MMSC	Malignant Melanoma Stem Cell

MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MRP	Multidrug Resistance Protein
MSH	Melanocyte Stimulating Hormone
MT	microtubules
Mt	Mitochondria
MTA2	MetTastasis-Associated protein 2
mTOR	Mammalian Target Of Rapamycin
MUT	Mutated
N	Nucleus
NFKB	Nuclear Factor-kB
NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
NICD	Notch IntraCellular Domain
NK	Natural Killer
NM	Nodular Melanoma
NMRI-nu/nu	Spontaneous Mutant T-Cell Deficient Mice
NOD-SCID	Non-Obese Diabetic Severe Combined Immunodeficiency
NOS2a	Inducible Nitric Oxigen Synthase
NP40	Nonyl Phenoxylpolyethoxylethanol
NRAS	Nuroblastoma RAS viral (v-ras) oncogene homolog
NSAID	Nonsteroidal Anti-Inflammatory Drugs
Oct3/4	Octamer-binding proteins Oct3/4
P	Phosphate
Pax3	Paired Box 3
PBMC	Peripherical Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Programmed Death-1
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDK1	Phosphatidylinositol-Dependent Kinase 1
PD-L1	Programmed Death Ligand -1
PE	PhycoErythrin
PerCP	Peridinin Chlorophyll Protein
PFA	Paraformaldehyde
PI3K	Phospholnositide-3 Kinase
PIP2	Phosphatidylinositol (4,5) bisphosphate
PIP3	Phosphatidylinositol (3,4,5) triphosphate
PMSF	PhenylMethaneSulphonylFluoride
POS	Positive
PS	PreSenilins
Pt	Patient
PTCH1	Patched 1
PTEN	Phosphate and TENsin homolog
PTGS2	Prostaglandin-endoperoxide synthase 2
Pygo	Pygopus

qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
R.T.	Room Temperature
Raf	RAS viral (v-raf) oncogene homolog
RAS	RAS viral (v-ras) oncogene homolog
RASSF-1	Ras Association domain-Family Member 1
Rb	Retinoblastoma
RGP	Radial Growth Phase
RNA	Ribonucleic acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species (ROS)
RPMI	Roswell Park Memorial Institute
RQ	Relative Quantification
S	Spheres
SAS	Statistical Analysis System
SCF	Stem Cell Factor
SCID	Severe Combined ImmunoDeficient
SCM	Stem Cell Medium
SCs	Stem Cells
SD	Standard Deviation
SDF-1	Stromal derived Factor-1
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
SEER	Surveillance, Epidemology and End Results
SFA	Sphere Forming Assay
SHC	Src Homolgous and Collagen protein
SHH	Sonic Hedgehog
siRNA	Small Interfering RNA
SKI	Sloan Kettering institute gene
SMAD-	Small Mother Dgainst Decapentaplegic -
SMOH	Smoothened
SNAI2	Snail homolog 2
SNP	Single Nucelotide Polymorphism
Sox10	Sex-determining region Y-box 10
Sox2	Sex-determining region Y-box 2
SP	Side Population
SSEA-	Stage-Specific Embryonic Antigen 1
SSM	Superficial Spreading Melanoma
Stat3	Signal Transducer and Activator of Transcription 3
Su(Fu)	Suppressor of Fused
SYK	Spleen Tyrosine Kinase
TA	Temperature of Annealing
TAE	Tris-acetate-EDTA
T-ALL	T-cell Acute Lymphoblastic Leukaemia
TBE	Tris/Borate/EDTA
Tbx2	T-box transcription factor

TCF	T-Cell transcription Factor
TEM	Trasmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
TICs	Tumour Initiating Cells
TMAs	Tumour Associated MAccrophages
TMZ	Temozolomide
TNF	Tumour Necrosis Factor
TNM	Tumour-Nodes-Metastases
TP53	Tumour Protein p53
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TRP-2	Tyrosinase-Related Protein 2
Tx	Triton X-100
TYR	TYRosinase
TYRP1	TYRosinase-Related Protein 1
U	Ubiquitin
Ulc	Ulceration
UVR	UltraViolet Radiation
V-CAM	Vascular-Cell Adhesion Molecule
VEGF	Vascular Epidermal Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VGP	Vertical Growth Phase
WT	Wild type

LIST OF FIGURES

LIST OF TABLES

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Heterogeneous Phenotype of Human Melanoma Cells with *In Vitro* and *In Vivo* Features of Tumor-Initiating Cells

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Melanospheres, the melanoma cells that grow as nonadherent colonies and that show *in vitro* self-renewing capacity and multipotency, were selected from melanoma specimens or from melanoma cell lines. Melanospheres were highly tumorigenic, and intradermal injections in severe combined immunodeficient (SCID) mice of as few as 100 cells generated tumors that maintained tumorigenic potential into subsequent recipients. Primary and serially transplanted xenografts recapitulated the phenotypic features of the original melanoma of the patient. Melanoma cells cultured in the presence of fetal calf serum (FCS) were also tumorigenic in SCID mice, although with lower efficiency; these xenografts showed a homogeneous phenotype for the expression of melanoma-associated markers, Melan-A/Mart-1, HMB45, and MITF, and contained cells with features of fully differentiated cells. Melanospheres were heterogeneous for the expression of stem cell markers and showed a significantly enhanced expression of the Nanog and Oct3/4 transcription factors when compared with adherent melanoma cells. No direct and unique correlation between any of the examined stem cell markers and *in vivo* tumorigenicity was found. Taken together, our data provide further evidence on the heterogeneous nature of human melanomas and show that melanospheres and their corresponding tumors, which are generated *in vivo* in immunocompromised mice, represent a model to investigate melanoma biology.

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INTRODUCTION

The cancer stem cell theory proposes that tumors can be viewed as the result of an anomalous differentiation program of putative cancer stem cells (CSCs). Although probably representing a minor population of the whole tumor mass, CSCs are believed to constitute a unique cell subset responsible for tumor maintenance and growth (Reya *et al.*, 2001; Lee and Herlyn, 2007; Ailles and Weissman, 2007). However, the parent-to-progeny relationship between CSCs and the tumor bulk population does not necessarily reflect the well-conserved and predictable rules operating in normal tissue organization. Instead, because of the intrinsic genetic and epigenetic instability of cancer cells, it is more likely that tumor generation represents an aberrant and highly unstable differentiation program (Adams and Strasser, 2008; Odoux *et al.*, 2008). Moreover, recent data suggest that tumor heterogeneity can result from variability in the frequency, phenotype, and functional features of CSCs (Piccirillo *et al.*, 2009).

Although there is no definitive consensus on the phenotype and frequency of CSCs in the majority of human tumors, much experimental evidence supports the contentions that many tumors of both epithelial and nonepithelial origin have operationally defined CSCs (cells able to propagate tumors in immunodeficient mice) and that the presence of these CSCs affects tumor biology (Alison *et al.*, 2008; Dick, 2009; Jordan, 2009).

Melanoma, one of the most aggressive human cancers with metastatic lesions resistant to conventional therapies, is highly heterogeneous and shows a high degree of plasticity

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Abbreviations: ADH, adherent; CSC, cancer stem cell; FCS, fetal calf serum; NGFR, nerve growth factor receptor; SCM, stem cell medium; SCID, severe combined immunodeficient

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(Hendrix *et al.*, 2003; Mourad-Zeidan *et al.*, 2008). Recent evidence indicates that human melanomas contain different cell populations endowed with intrinsic chemoresistance (Frank *et al.*, 2005). Moreover, it has been shown that stem-associated markers such as CD133, CD166, and nestin are expressed by human melanomas (Klein *et al.*, 2007). Although the nature and frequency of CSCs in melanoma is controversial, it is likely that the CSC model can be applied to human melanoma, and different surface markers have been proposed to define melanoma cells with tumor-initiating potential (Fang *et al.*, 2005; Monzani *et al.*, 2007; Keshet *et al.*, 2008; Schatton *et al.*, 2008). A recent study using nonobese diabetic/severe combined immunodeficient (SCID) $Il2r\gamma^{-/-}$ mice for an *in vivo* assay of tumor initiation suggested a very high frequency of CSCs in melanoma; moreover, an analysis of a large panel of melanoma-related markers failed to find any correlation between a specific phenotype and tumor-initiating capacity *in vivo* (Quintana *et al.*, 2008). Thus, stem cells in melanoma are worthy of investigation, and our study evaluated the presence of cells showing *in vitro* and *in vivo* features of tumor-initiating cells in human metastatic melanomas.

RESULTS

Anchorage-independent, self-renewing melanospheres variably express stem-associated markers

Stem cell medium (SCM) supported the selection of cells growing as melanospheres in 8 of the 18 lymph node melanoma metastases used in this study (44% success rate). A similar percentage of melanosphere formation (32%) was achieved by starting from melanoma cell lines previously established as adherent cells in medium supplemented with fetal calf serum (FCS). MeJR8S and Me204S, derived from the adherent melanoma cell lines, and Me15888S and Me14346S, derived directly from lymph node metastasis, were used throughout this study. The self-renewing capacity of tumor spheres was assayed by sphere formation and clonogenic assays. Both tests gave consistent results, showing that all of the melanospheres showed a higher frequency of self-renewing cells than adherent melanomas (Table 1).

Melanospheres were cultured in differentiating media for cells of the mesenchymal lineage: chondrocytes, osteocytes, and adipocytes. Me204S was able to differentiate into all three cell types with similar efficiencies (Supplementary Figure S1 online). Melan-A/MART-1 expression, a marker of melanocyte differentiation, was evaluated after growing melanosphere-derived cells in the presence of 10% FCS. The corresponding adherent melanoma cell line Me204ADH did not show differentiation capability and maintained a high expression of Melan-A/MART-1. Similar differentiation rates were obtained for MeJR8S, Me15888S, and Me14346S (data not shown).

All of the generated melanospheres were negative for the hematopoietic marker CD45. Conversely, all of them were 100% positive for Melan-A/MART-1 and HMB45 melanocyte differentiation proteins (Figure 1a). The expression of CD146 and GD3 was variable, with virtually all Me14346S cells staining negative for GD3 and a very low percentage staining positive for CD146 (Figure 1a). Melanosphere cells also expressed several embryonic stem cell markers, and all four of the melanosphere lines were almost 100% positive for CD9, SOX2 Oct3/4, and Nanog proteins whereas a very low percentage of cells (or none at all) expressed SSEA-1 and SSEA-4. A low rate of expression was observed for Musashi-1, with 13–16% of Me15888S and Me204S cells staining positive (Figure 1b and Supplementary Figure S2 online). The expression of stem-related markers used to identify adult stem cells of normal and malignant tissue (CD20, CD24, CD44, CD73, CD133, CD166, nerve growth factor receptor (NGFR), and nestin) was examined (O'Brien *et al.*, 2009). NGFR and nestin were included because of their expression in cells of the neural crest (Lee *et al.*, 2007). All the melanosphere lines were negative for CD20 and positive for CD44 and nestin. Conversely, CD24, CD73, CD166, NGFR, and CD133 markers were expressed in different percentages of cells in all the melanosphere lines. In Me15888S, a majority of cells were CD133−, CD166−, and CD73+, and this melanosphere line had the highest percentage of NGFR-positive cells (Figure 1c).

The expression of all the above markers was also assessed in Me204ADH, JR8ADH, and Me14346ADH and in seven additional pairs of adherent and melanosphere melanoma lines. Among the examined markers, only the expression of the stem-related proteins Nanog and Oct4 was significantly

Table 1. Self-renewal of melanospheres (S) and melanoma-adherent cells (ADH)

Cell type	% of melanospheres	
	SFA[1]	Clonogenic assay[2]
Me204		
S	54 ± 7.15[3]	52.7 ± 5.6
ADH	10.7 ± 8.2	4.4 ± 0.3
MeJR8		
S	25.6 ± 1.1	18.5 ± 2.1
ADH	8.2 ± 3.8	6.4 ± 0.2
Me14346		
S	26.9 ± 4.1	11.8 ± 4.2
ADH	0.9 ± 1.1	0
Me15888[4]		
S	14 ± 8.2	5.8 ± 1.8

Abbreviation: SFA, Sphere Formation Assay.
[1]Percentage of melanospheres per 100 cells.
[2]Percentage of clones per total single cells seeded.
[3]Mean ± SD calculated on the bases of three independent experiments.
[4]Fresh melanoma cell suspension derived from lymph node melanoma metastasis no. 15888 failed to establish an adherent melanoma cell line, and thus the adherent counterpart of Me15888S is not available for analysis.

Figure 1. Immunophenotype of melanospheres. Immunofluorescence and FACS analysis of CD45 and melanoma-associated markers. Shown are the (**a**) embryonic, (**b**) adult, and (**c**) stem cell markers expressed by melanospheres generated from melanoma cell lines (Me204S and JR8S) and from fresh melanoma specimens (Me15888S and Me14346S). Thin black histograms represent controls stained with negative isotopic antibodies; filled histograms correspond to specific markers; numbers indicate the percentage of positive cells. Histograms are representative of three independent experiments.

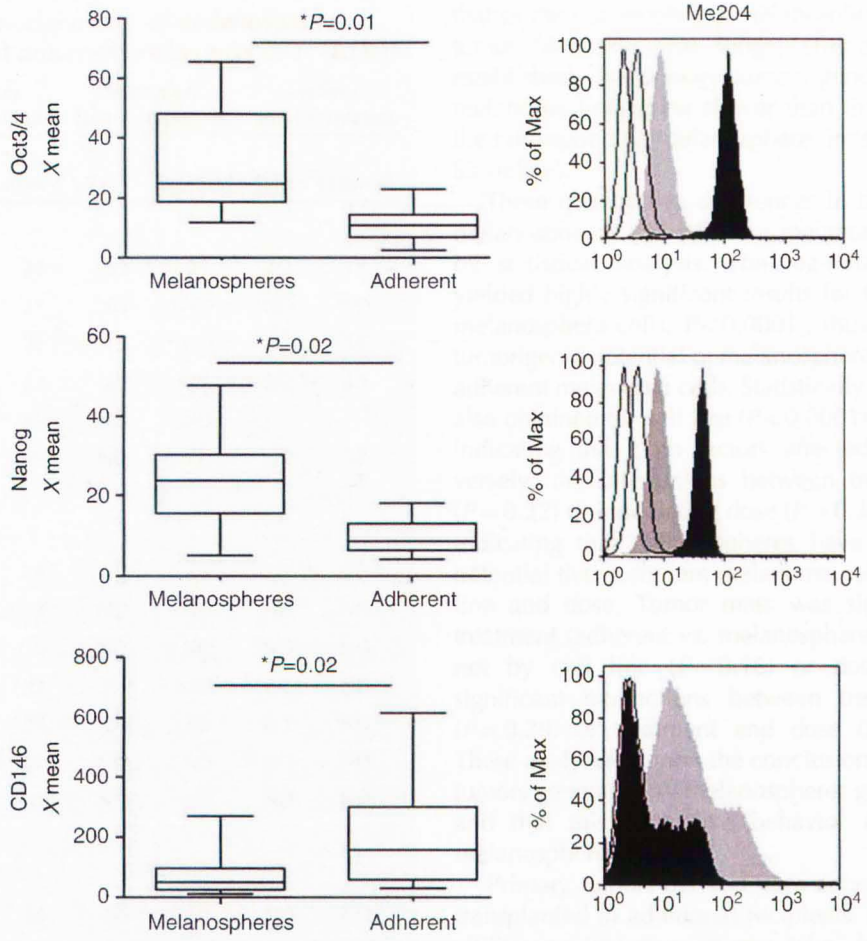

Figure 2. Oct3/4, Nanog, and CD146 are modulated in melanospheres when compared with adherent cells. The left column shows the box-and-whisker plots for the markers that were differentially expressed in melanospheres when compared with adherent cells. The fluorescence intensity (X mean) on the *y* axis equals the X mean (marker) minus the X mean (isotype control). Values are calculated as the mean of three independent experiments. Histograms of the differential expression of Oct3/4, Nanog, and CD146 in Me204S (black) and Me204ADH (gray) are reported. Lines and filled histograms correspond to isotype controls and specific antibodies, respectively (*P*-values evaluated with unpaired *t*-test). In all, 11 melanoma adherent cell lines and 11 melanospheres were analyzed.

enhanced in melanospheres when compared with adherent cells, and only CD146 was significantly downregulated (Figure 2).

Real-time PCR clearly showed that ABCB1, ABCB5, and ABCG2 genes were expressed by melanocytes. Moreover, all of the melanosphere and adherent cell lines were positive for ABCG2, whereas they variably expressed ABCB1 and ABCB5 (Supplementary Table S1 online).

Melanospheres are endowed with enhanced tumorigenicity when compared with adherent melanoma cells

The tumorigenicity of melanospheres was assessed by evaluating their capacity to form melanomas when intradermally injected into SCID mice. At a dose of 10^4 cells, all of the melanosphere lines generated fast-growing tumors in 100% of injected mice within 14–48 days. Notably, all of the melanosphere lines could form tumors when as few as 100 cells were injected per mouse, and two of them (Me15888S and MeJR8S) formed tumors in 100% of mice even at this low dose. Primary xenografts were then serially transplanted into subsequent animal hosts. Secondary and tertiary tumors developed in 100% of injected mice; interestingly, serial tumors grew as fast or faster than their corresponding primary engraftment (Table 2 and Supplementary Figure S3 online).

To provide evidence that the melanoma xenografts generated by melanospheres may have had a clonal origin, SCID mice were inoculated with escalating doses of cells derived from melanospheres generated from a single melanoma clone, P1E8S. Tumor take, latency, and secondary tumor formation were all similar to those of the parental melanosphere bulk population (JR8S) from which the clone was generated (Supplementary Table S2 online).

Because melanospheres endowed with strong tumorigenic potential could have been selected from melanoma cells adapted to grow in FCS, and because even the adherent melanoma lines had a limited but detectable *in vitro* self-renewal capacity, Me204ADH, MeJR8ADH, and Me14346ADH cells were inoculated in SCID mice to evaluate the extent to which they possess tumor-initiating

Table 2. *In vivo* tumorigenicity of melanosphere-forming cells (S) and adherent melanoma cells (ADH)

		Primary tumor formation		Secondary tumor formation		Tertiary tumor formation	
Cell type	Cell number[1]	Take[2]	Latency[3]	Take[2]	Latency[3]	Take[2]	Latency[3]
MeJR8							
S	10^4	5/5	22	2/2	25	2/2	18
	10^3	5/5	29	ND	ND	ND	ND
	10^2	5/5	22	ND	ND	2/2	35
ADH	10^4	5/5	22	2/2	25	2/2	30
	10^3	4/5	49	ND	ND		
	10^2	3/5	49	ND	ND	2/2	50
Me204							
S	10^4	4/4	14	2/2	36	2/2	28
	10^3	5/5	22	ND	ND	ND	ND
	10^2	2/5	39	ND	ND	ND	ND
ADH	10^5	ND	ND	2/2	36	0/2	ND
	10^4	5/5	25	2/2	60	0/2	ND
	10^3	5/5	67	ND	ND	ND	ND
	10^2	0/5	ND	ND	ND	ND	ND
Me14346							
S	10^4	5/5	48	2/2	30	ND	ND
	10^3	4/5	92	3/3	40	2/2	30
	10^2	3/5	123	3/3	40	3/3	40
	5×10^1	ND	ND	ND	ND	3/3	57
ADH	10^6	2/2	19	0/1[4]	ND	ND	ND
	10^4	5/5	77	0/2[4]	ND	ND	ND
	10^3	4/5	77	ND	ND	ND	ND
	10^2	3/5	123	0/2[4]	ND	ND	ND
Me15888[5]							
S	10^4	5/5	33	3/3	35	3/3	35
	10^3	5/5	33	ND	ND	ND	ND
	10^2	5/5	53	ND	ND	ND	ND

Abbreviation: ND, not done.
[1]Number of injected cells.
[2]Number of tumors formed per number of injections.
[3]Time from injection to tumor measurability. Median values are reported. Observation time is 200 days.
[4]Observation time is 60 days.
[5]Fresh melanoma cell suspension derived from lymph node melanoma metastasis no. 15888 failed to establish an adherent melanoma cell line, and thus the adherent counterpart of Me15888S is not available for analysis.

capacity *in vivo*. As expected, tumor take decreased with diminishing numbers of injected cells; the efficiency of primary tumor formation was lower when compared with that of the corresponding melanospheres, and the latency of tumor formation was longer (Table 2). Moreover, once established, the primary tumors generated by the adherent melanoma lines grew slower than the tumors generated by the corresponding melanosphere lines (Supplementary Table S3 online).

These quantitative differences in the *in vivo* behavior of melanospheres and adherent melanoma cells were assessed by statistical analysis. The log-normal regression model yielded highly significant results for treatment (adherent vs. melanosphere cells; $P<0.0001$), thus confirming the higher tumorigenic potential of melanospheres when compared with adherent melanoma cells. Statistically significant results were also obtained for cell line ($P<0.0001$) and dose ($P<0.0001$), indicating that both factors affected tumorigenicity. Conversely, the interactions between treatment and cell line ($P=0.22$) or inoculating dose ($P=0.30$) were not significant, indicating that melanospheres have a higher tumorigenic potential than adherent melanoma cells, independent of cell line and dose. Tumor mass was significantly affected by treatment (adherent vs. melanosphere cells; $P=0.0045$) but not by cell line ($P=0.16$) or dose ($P=0.35$), and no significant interactions between treatment and cell line ($P=0.29$) or treatment and dose ($P=0.79$) were found. These analyses support the conclusion that, once established, tumors generated by melanospheres grow more aggressively, and that this aggressive behavior is a function of their melanosphere origin.

Primary tumors derived from adherent cells were serially transplanted in additional recipients. Differences in translatability, evaluated as tumor take, between adherent and melanosphere-derived primary xenografts emerged in tertiary tumor formation with Me204ADH- and Me14346ADH-derived tumors failing to grow. Me14346ADH xenografts showed very poor viability (Table 2), and viable tumor cells were only obtained from tumors generated by the injection of 10^6 melanoma cells. However, at the same cell dose, no serial engraftment was successfully established. Of note and at difference from Me204ADH and Me14346ADH, MeJ R8ADH showed transplantation efficiency equal to its autologous MeJR8S, and 100% tumor take occurred with all four tumors growing in tertiary recipients.

Xenografts generated by melanospheres recapitulate the phenotypic features of the original human melanoma

Histological evaluation confirmed that human melanoma xenografts generated in SCID mice by melanospheres consisted of cells showing the typical morphology of melanoma cells: eosinophilic cytoplasm, irregular nuclei, and prominent nucleoli. Xenografted tumors expressed the specific melanoma markers HMB45, Melan-A/Mart-1, and MITF-1. Moreover, although melanospheres were homogeneously positive for Melan-A/MART-1, this marker was heterogeneously expressed in the growing tumor mass; i.e., Melan-A/Mart-1-negative melanoma cells could be found. In general, this heterogeneity, as well as the levels of MITF and HMB45, mirrored that of the patient's original tumor (Figure 3a). Furthermore, the similarity between the

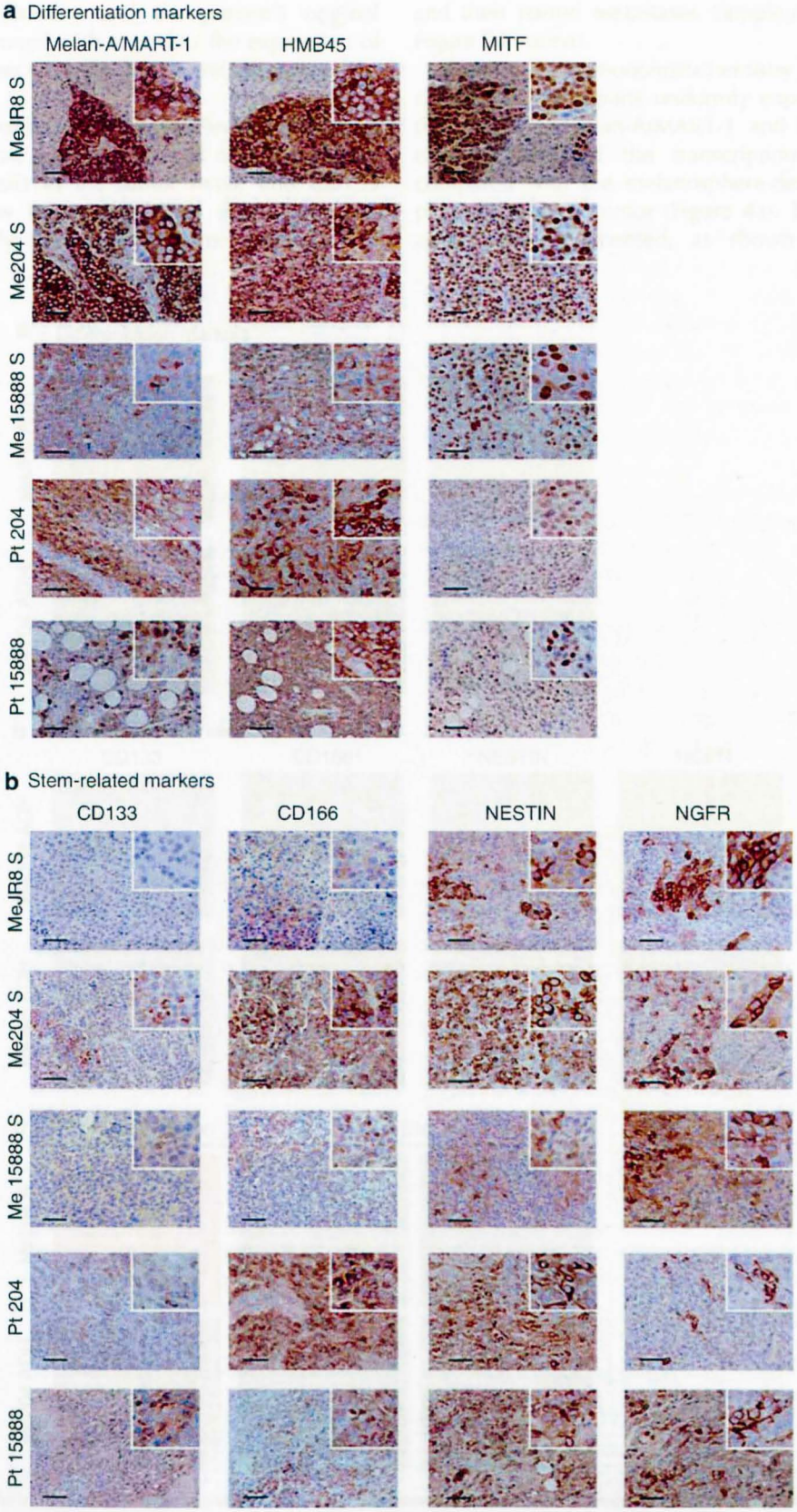

Figure 3. Melanospheres are tumorigenic and reproduce human melanoma in immunocompromised mice. Immunohistological staining of (**a**) melanoma-associated and (**b**) stem-related markers in xenograft tumors generated by intradermal injection of melanosphere-derived cells and in lymph node metastatic melanomas of patient 204 and patient 15888. Internal scale markers are indicated in each panel. For melanosphere-derived xenografts, reported images are representative of eight independently analyzed xenografts; for patients' tumors, images are representative of two non-consecutive tumor sections. Scale bar = 100 μm.

melanosphere-derived tumors and the patient's original tumor was also maintained with regard to the expression of the stem-related markers CD133, CD166, NGFR, and nestin (Figure 3b).

The stem-related markers showed an elevated degree of variability in expression, with CD166 and nestin expressed by the majority of cells in the tumor mass, and CD133 expressed by very few tumor cells. This distribution was confirmed by the analysis of 10 primary melanoma lesions and their paired metastases (Supplementary Table S4 and Figure S4 online).

Conversely, immunohistochemistry showed that adherent cell-derived xenografts uniformly expressed the differentiation markers, Melan-A/MART-1 and HMB45, and had an elevated level of the transcription factor MITF when compared with the melanosphere-derived tumors and the patient's original tumor (Figure 4a). These xenografts were also heavily pigmented, as shown by melanin-specific

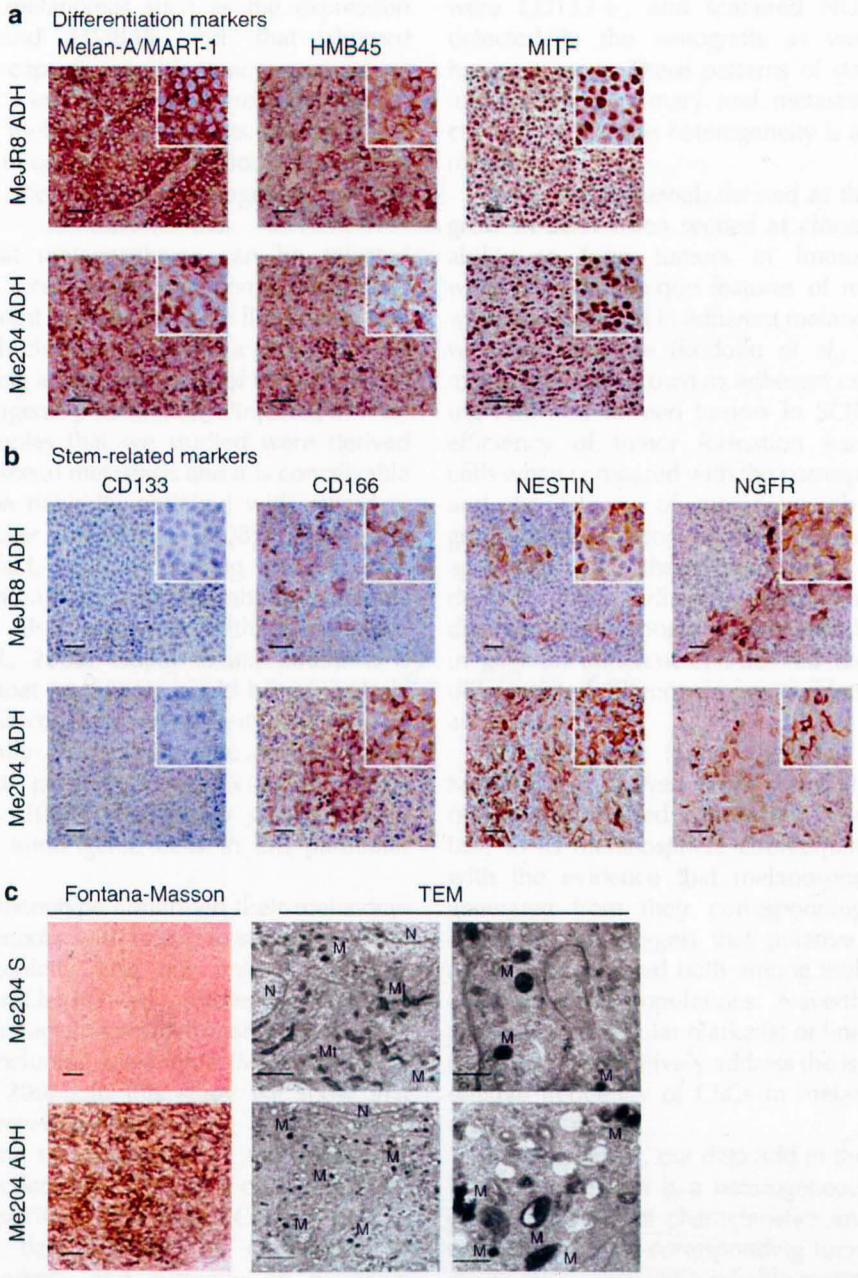

Figure 4. Immunohistochemistry and ultrastructural analysis of xenografted tumors. Immunohistochemical staining of (**a**) melanoma-associated and (**b**) stem-related markers was performed on xenografts generated by intradermal injection of adherent melanoma cells Me204 and MeJR8. For each cell line, reported images are representative of eight independently analyzed xenografts. Internal scale markers are indicated in each panel. (**c**) Melanin staining and transmission electron microscopy (TEM) analysis. Fontana-Masson melanin-specific staining (**c**, left panels) and TEM (**c**, middle and right panels) on melanoma xenografts derived from the injection of Me204 melanospheres (upper panels) or Me204-adherent cells (lower panels). M, melanosomes; Mt, mitochondria; N, nucleus. Scale bar = 100 μm. For TEM, left panel scale bar = 2 μm and right panel scale bar = 500 nm.

staining (Figure 4c, left panel). High numbers of melanosomes, mostly in their terminal differentiation phase, were detected by electron microscopy in these adherent cell-derived tumors, whereas only a few, mainly immature melanosomes, were found in melanosphere-derived tumors (Figure 4c, right panel).

DISCUSSION

In this study we show that from lymph node metastatic lesions we isolated, by selective *in vitro* culture, melanoma cells growing as melanospheres, which had distinctive features of melanomas such as the expression of Melan-A/Mart-1 and HMB45 and that showed *in vitro* self-renewing capacity, multipotency, and strong tumorigenic potential in immunocompromised mice. Moreover, the human melanoma xenografts, generated in immunocompromised mice by the injection of melanospheres, recapitulated the original heterogeneity of the patient's tumors.

Our data show that melanospheres can be selected with high efficiency from bulk melanoma cells and from established adherent melanoma cell lines, and that these cells show a high clonogenicity and a strong *in vivo* tumorigenicity, suggesting a high frequency of cells endowed with or acquiring tumorigenic potential once injected *in vivo*. All the melanoma samples that we studied were derived from lymph nodes or visceral metastasis and it is conceivable that metastases may be naturally enriched with regard to tumorigenic cells (Croker and Allan, 2008; Chiang and Massague, 2008); in fact, cells undergoing an epithelial–mesenchymal transition and acquiring the ability to invade and metastasize are also endowed with tumorigenic properties (Mani *et al.*, 2008; Gupta *et al.*, 2009). It is tempting to speculate that metastases could be particularly enriched for tumorigenic cells and, because it is known that melanomas are generally highly metastatic and endowed with a high degree of plasticity (Hendrix *et al.*, 2003; Mourad-Zeidan *et al.*, 2008), this theory could explain the high frequency of tumorigenic cells in this particular human cancer.

The melanosphere phenotype confirmed their melanocytic origin, was heterogeneous with regard to stem-associated markers, and tumorigenicity was not linked with any particular phenotypic profile. Recently, studies have stressed the role of CD133 as a cancer stem cell marker in a set of human solid tumors, including melanoma (Monzani *et al.*, 2007; O'Brien *et al.*, 2007). In this study we show that melanospheres homogeneously expressing CD133 were endowed with the same tumorigenicity as melanospheres with only a limited percentage of CD133+ cells. Similarly, we did not detect any specific enrichment of CD20+ cells in melanospheres. These data parallel the complexity of characterizing CSC markers, and currently no definitive consensus has been reached on the CSC phenotype for melanoma (Fang *et al.*, 2005; Frank *et al.*, 2005; Monzani *et al.*, 2007; Keshet *et al.*, 2008; Schatton *et al.*, 2008; Quintana *et al.*, 2008) or indeed for other solid tumors or normal tissues (Dalerba *et al.*, 2007; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Becker *et al.*, 2008; Shmelkov *et al.*, 2008; Cheng *et al.*, 2009; Sun *et al.*, 2009).

The histological features of the xenografts generated by melanospheres clearly resembled those of human melanomas, thus recapitulating the original tumor heterogeneity. Although the melanospheres were homogenously positive for Melan-A/MART-1, this marker was heterogeneously expressed in melanoma xenografts, and in general, the expression patterns of melanoma-associated markers and stem-related markers mirrored those of the patient's original tumors. In particular, very few cells were CD133+, and scattered NGFR+ cell nests were detected in the xenografts as well as in the original human tumors. These patterns of staining were also found in a panel of primary and metastatic paired melanomas, confirming that this heterogeneity is a general trait of human melanomas.

In vitro self-renewal, defined as the ability of the cells to grow in SCM when seeded at clonogenic density, and the ability to form tumors in immunocompromised mice were not only unique features of melanospheres but they were also detected in adherent melanoma cells. In agreement with the literature (Rodolfo *et al.*, 1988), we found that melanoma cells grown as adherent cells in medium containing FCS also formed tumors in SCID mice. However, the efficiency of tumor formation was lower for adherent cells when compared with the corresponding melanospheres, and the latency of tumor growth was delayed in the group of tumors derived from adherent melanoma cells. In agreement with these quantitative differences, xenografts derived from adherent melanoma cells qualitatively diverged from xenografts generated by melanospheres and, in general, adherent cell-derived tumors showed a highly differentiated phenotype, as evidenced by ultrastructural analysis.

Of note, we found that the melanoma cell line MeJR8ADH, derived from visceral metastasis (stage IV melanoma), showed over serial *in vivo* passages equal tumor take to its melanosphere counterpart. These data, together with the evidence that melanospheres could have been generated from their corresponding adherent melanoma cells, strongly suggest that putative melanoma stem cells are likely contained both among melanospheres and adherent melanoma populations. Nevertheless, because of the absence of molecular marker(s) or lineage tracing *in vivo*, our data did not definitively address the issue of the presence and relative frequency of CSCs in melanosphere and adherent melanoma cells.

Taken together, our data add to the existing evidence that human melanoma is a heterogeneous cancer composed of cells with different characteristics and suggest that melanospheres and their corresponding tumors in immunocompromised mice represent a reliable model to analyze melanoma biology. A better understanding of the fate of melanospheres inoculated *in vivo*, and their response to stimuli deriving from the tumor stroma, will offer the possibility to better understand the mechanisms driving melanoma formation and progression.

MATERIALS AND METHODS

Melanoma cell cultures

Lymph node melanoma metastases were collected upon patients' informed written consent. The melanoma adherent cell lines Me204ADH and Me14346ADH were established as reported (Vallacchi *et al.*, 2008). The melanoma cell line JR8ADH, derived from a melanoma lung metastasis, was kindly provided by Dott G. Zuppi, Istituto Regina Elena, Rome. Fresh melanoma cell suspension derived from lymph node melanoma metastasis no. 15888 failed to establish an adherent melanoma cell line. To generate Me15888S and Me14346S melanospheres, fresh melanoma cells were seeded at clonal density (2,500–5,000 cells cm^{-2}) in 75-cm^2 flasks in SCM consisting of DMEM/F-12 (Lonza, Valais, Switzerland), supplemented with N2 supplement (Invitrogen, Carlsbad, CA). Melanospheres Me204S and JR8S were derived by plating melanoma cells grown as a monolayer (Me204ADH and MeJR8ADH) in SCM. Clone P1E85 was derived by plating primary melanospheres of JR8S at clonal density (1 cell per well) in SCM. The study was conducted according to the Declaration of Helsinki Principles.

Sphere formation assay and clonogenic assay

For the sphere formation assay, 100 single, viable melanoma cells were plated in 500 μl of SCM in poly-L-lysine (Sigma, St Louis, MO), coated overnight in a 48-well plate and melanospheres were counted after 5–8 days. For the clonogenic assay, 100 μl of SCM containing 2.5 cells ml^{-1} was seeded in 96-well plate. After 2–4 hours, wells were examined under the microscope and wells containing a single cell were followed for sphere formation over the following days.

Flow cytometry

The primary antibodies, anti-human CD24, CD44, CD73, CD146, CD166, and NGFR (CD271) were all from Becton Dickinson (Franklin Lakers, NJ). In addition, anti-human CD133 (clone C293; Miltenyi, Bergisch Gladback, Germany); anti-human Nestin (R&D, Minneapolis, MN); anti-human Melanosome (HMB45, Dako, Glostrup, Denmark) and anti-human Melan-A/MART-1 (clone M2-7; Kawakami *et al.*, 1997); and anti-human Musashi-1, Sox2, Oct3/4, and Nanog (R&D) were used. For intracellular staining, Cytofix/Cytoperm and Perm/Wash buffer (Becton Dickinson) were used; nuclear antigens were analyzed in cells fixed with 4% paraformaldehyde and permeabilized with TX-100 0.1% in phosphate-buffered saline for 30 minutes at room temperature. Data were acquired by FACScalibur (Becton Dickinson) and analyzed using FlowJo, V 8.5.2, Tree Star, Ashland, OR.

Real-time PCR

RNA extraction and digestion with DNase were carried out using the RNAqueous-4PCR Kit (Ambion, Carlsbad, CA). Complementary DNA synthesis was performed with the High-Capacity complementary DNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). The real-time PCR reaction was performed with inventoried assays, using the ABI 7900HT instrument (Applied Biosystems).

In vivo tumorigenicity

SCID mice, 7 weeks old, were intradermally injected with melanoma cells, resuspended in phosphate-buffered saline, and mixed with Matrigel (Becton Dickinson) in a 1:1 ratio (Charles River, Wilmington, MA). Viability of the injected cells was determined using the Trypan blue exclusion test. To perform serial engraftments, mice bearing tumors of approximately 500 mg were killed, their tumors were dissociated, and viable single cells were re-injected into SCID mice. The experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan, according to the institutional guidelines.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (1–2 μm) were stained according to the standard procedures (Vallacchi *et al.*, 2008; Klein *et al.*, 2007) with the following primary mouse anti-human antibodies: anti-melanosome, 1:50 (HMB45, Dako); anti-Melan-A, 1:50 (Dako clone A103); anti-MITF, 1:200 (clone 34CA5; Novocastra Laboratories, New Castle, UK); anti-nestin, 1:200 (Chemicon International, Bilerica, MA); anti-CD166, 1:40 (clone MOG/07; Novocastra Laboratories); anti-NGFR, 1:50 (Novocastra Laboratories); and anti-CD133, 1:25 (clone AC133; Miltenyi). Stained sections were analyzed by optical microscope (Nikon Eclipse 600, Nikon, Tokyo, Japan). Fontana-Masson silver staining was performed on 6-μm deparaffinized and hydrated sections according to the standard procedures.

Transmission electron microscopy analysis

Tumors were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.12 M phosphate buffer. All samples were post-fixed with 1% OsO_4 in cacodylate buffer, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections (60 nm), obtained with an ultra microtome (Ultracut E; Reichert-Jung, Depew, NY), were doubly stained with uranyl acetate and lead citrate and then examined by transmission electron microscope CM 10 Philips (FEI, Eindhoven, The Netherlands).

Statistical analyses

The differences in tumorigenicity (tumor take and latency) and in the tumor mass of the xenografts generated by melanospheres and adherent cells were statistically evaluated. For all the analyses, multiple regression models were used: a log-normal model for "time-to-event" data to evaluate differences in the tumorigenicity, and a generalized linear model for normal data in the case of tumor mass. The statistical assumptions were verified, and the log-normal model was chosen as being the best fit among a number of parametric alternatives (exponential, Weibull, and log-logistic models). The model predictors were treatment (melanospheres and adherent melanoma cells), cell line (Me204, MeJR8, and Me14346), and dose (10^4, 10^3, and 10^2). When treatment was the factor of experimental interest, cell line and dose represented study design-related covariates. All these factors were categorical, and thus coded within the models by indicator (0–1) dummy variables. A search for interactions between treatment and cell line or dose was also performed. Analyses were performed with SAS (Cary, NC); two-sided *P*-values of <0.05 were considered statistically significant. Row outputs of the analyses are included in the Supplementary Material.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Table 1s . Expression of ABC transporter genes in melanospheres (S) or adherent melanoma cells (ADH) evaluated by Real Time PCR

Cell type		Transporters					
		ABCB1		ABCB5		ABCG2	
		[1]ΔC_t	[2]RQ	[1]ΔC_t	[2]RQ	[1]ΔC_t	[2]RQ
Me204	**S**	12,96	0,66	[3]-	0,00	11,17	1,10
	ADH	14,07	0,30	[3]-	0,00	9,78	2,88
MeJR8	**S**	[3]-	0,00	[3]-	0,00	12,25	0,52
	ADH	17,25	0,03	18,01	0,00	11,21	1,08
Me14346	**S**	15,70	0,10	8,48	0,22	12,34	0,49
	ADH	11,72	1,56	11,98	0,02	12,22	0,53
Me15888[4]	**S**	5,61	107,35	[3]-	0,00	12,09	0,58
Melanocytes		12,36	1,00	6,27	1,00	11,31	1,00

[1] ΔC_t values calculated as [C_t (target gene)- C_t (GAPDH RNA)]
[2] RQ values calculated using the equation $RQ=2^{-\Delta\Delta Ct}$. $\Delta\Delta C_t$ values were calculated by subctracting the ΔC_t of the calibrator from the ΔC_t value of each target.
[3] Absent genes defined as genes with ΔC_t above 25 cycles.
[4] Fresh melanoma cell suspension derived from lymph node melanoma metastasis #15888 failed to establish an adherent melanoma cell line, thus the adherent counterpart of Me15888S is not available for analysis

Table 2s *In vivo* tumorigenicity of the melanosphere clone P1E8 S.

Cell type	Cell Number[1]	primary tumor formation		secondary tumor formation	
		Take[2]	Latency[3]	Take[2]	Latency[3]
MeP1E8S	10^4	5/5	28	2/2	26
	10^3	5/5	28	nd	
	10^2	5/5	39	2/2	29

[1] Number of injected cells.
[2] Number of tumours formed / number of injections.
[3] Time from injection to tumour measurability. Median values are reported. Experiments were followed up to 200 days.
nd not done.

Table 3s. Growth rate of tumors generated in SCID mice by melanosphere (S) or adherent melanoma cells (ADH).

Cell type	Primary Tumors			
	Cell dose	Observation day	Tumor Mass (mg)[1]	
			S	ADH
MeJR8	10^4	42	264,1 ± 70,1	257,9 ± 67,1
	10^3	63	590 ± 130,4	363,8 ± 110,7
	10^2	85	1104,8 ± 256,5	538,8 ± 82,5
Me204	10^4	105	1021,8 ± 65,2	638,1 ± 82
	10^3	105	869,3 ± 150,4	256 ± 48,6
	10^2	83	602,5 ± 197,5	NA[2]
Me14346	10^4	85	643,5 ± 160,8	248,2 ± 100,2
	10^3	120	523,9 ± 170,7	926,7 ± 178,3
	10^2	140	418,2 ± 182,3	274,3 ± 211,8

[1]Tumor mass calculated as mean ± SE of the masses of the growing tumors.
[2]NA, no tumors grew in the primary recipients

Table 4s. Expression of stem cell markers on paired primary and metastatic melanoma

Patient		NGFR	CD133	Primary tumors			Metastasis site
				Clark Level	Thickness	Ulceration	
1	P	-	-	IV	19 mm	yes	
	M	**	-				Lymph node
2	P	*	*	IV	5,3 mm	no	
	M	**	-				Lymph node
	M	*	-				Soft tissues
3	P	*	*	IV	9,6 mm	yes	
	M	*	*				Soft tissues
4	P	*	-	V	7,5 mm	yes	
	M	**	*				Lymph node
5	P	*	*	IV	4,9 mm	yes	
	M	**	-				Lymph node
6	P	*	*	IV	12 mm	yes	
	M	*	-				Lymph node
7	P	-	-	III	8 mm	no	
	M	-	-				Lymph node
8	P	**	-	III	3,6 mm	no	
	M	**	-				Soft tissues
9	P	**	*	IV	8,2 mm	yes	
	M	**	**				Soft tissues
10	P	**	*	IV	>4 mm	yes	
	M	**	**				Soft tissues

P: Primary tumors; M: Metastasis; -:<5%; *:<20% of positive cells; **:<70% of positive cells; ***: >70% of positive cells. Positive cells were evaluated in 10 random fields.

Legends to the supplementary figures

Figure 1S. Mesenchymal differentiation of melanospheres and melanoma adherent cells. (A) and (B) Me204S and Me204ADH cells were induced to differentiate in osteogenic, adipogenic chondrogenic media as indicated in the Human Mesenchymal Stem Cell Functional Identification Kit (R&D). Differentiated cells were then stained for osteocalcin (green, Alexa 488), FABP4 (red, TRITC) or Aggrecan (red, TRITC), and with Hoechst33342 (upper and lower row in A and B). After washing, cover slips were mounted with 20% Mowiol (Sigma) and cells were analyzed by fluorescence microscopy with a Nikon Eclipse 600 (Nikon, Dusseldorf, Germany). No expression of any of the differentiation markers was detected in adherent cells. Melan-A/MART-1 staining was also performed after culturing Me204S and Me204ADH in medium with serum. (C) Histogram shows the percentage of Me204S (grey columns) and Me204ADH (black column) cells expressing the indicated differentiation marker counted in ten 20X random fields. Three independent experiments were performed and each experiment was performed in triplicate. Scale bar = 100 μm.

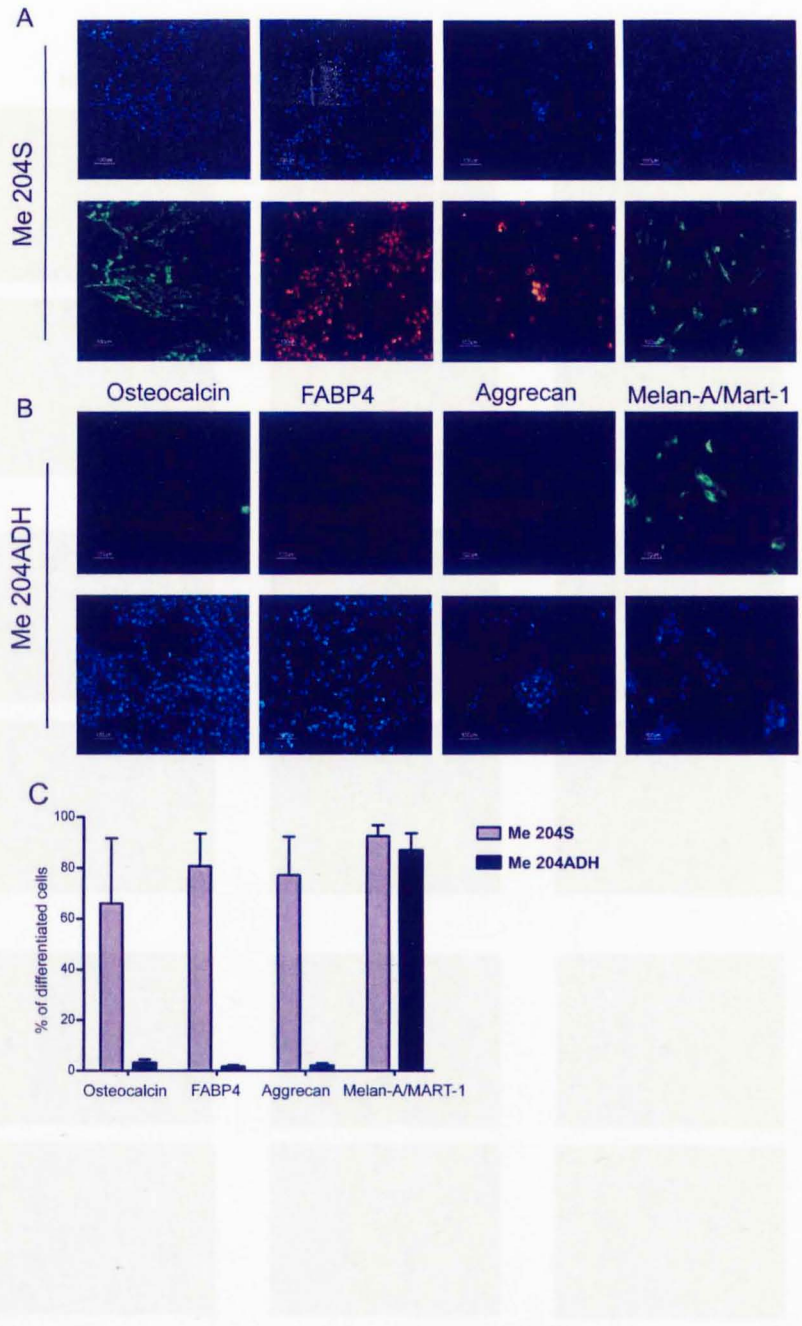
Figure 2S. Expression of Oct3/4 and Nanog in Me14346S. Me14346S were dissociated and 10^5 melanoma single cells seeded on microscope slide by cytospin. Staining was performed using Oct3/4 or Nanog antibodies (green, Alexa 488) and Hoechst33342, according to protocol indicated in the "Embryonic Stem Cell Markers Antibody Panel Plus" (R&D). After washing, cover slips were mounted with 20% Mowiol (Sigma) and cells were analyzed by fluorescence microscopy with a Nikon Eclipse 600 (Nikon) Images at 20X (scale bar 100μ) and 40X (scale bar 10μ) have been reported. Merge of blue and green images showed that nearly the totality of cells expressed Oct3/4 or Nanog in the nucleus. White arrows indicate the few cells not displaying any positivity for

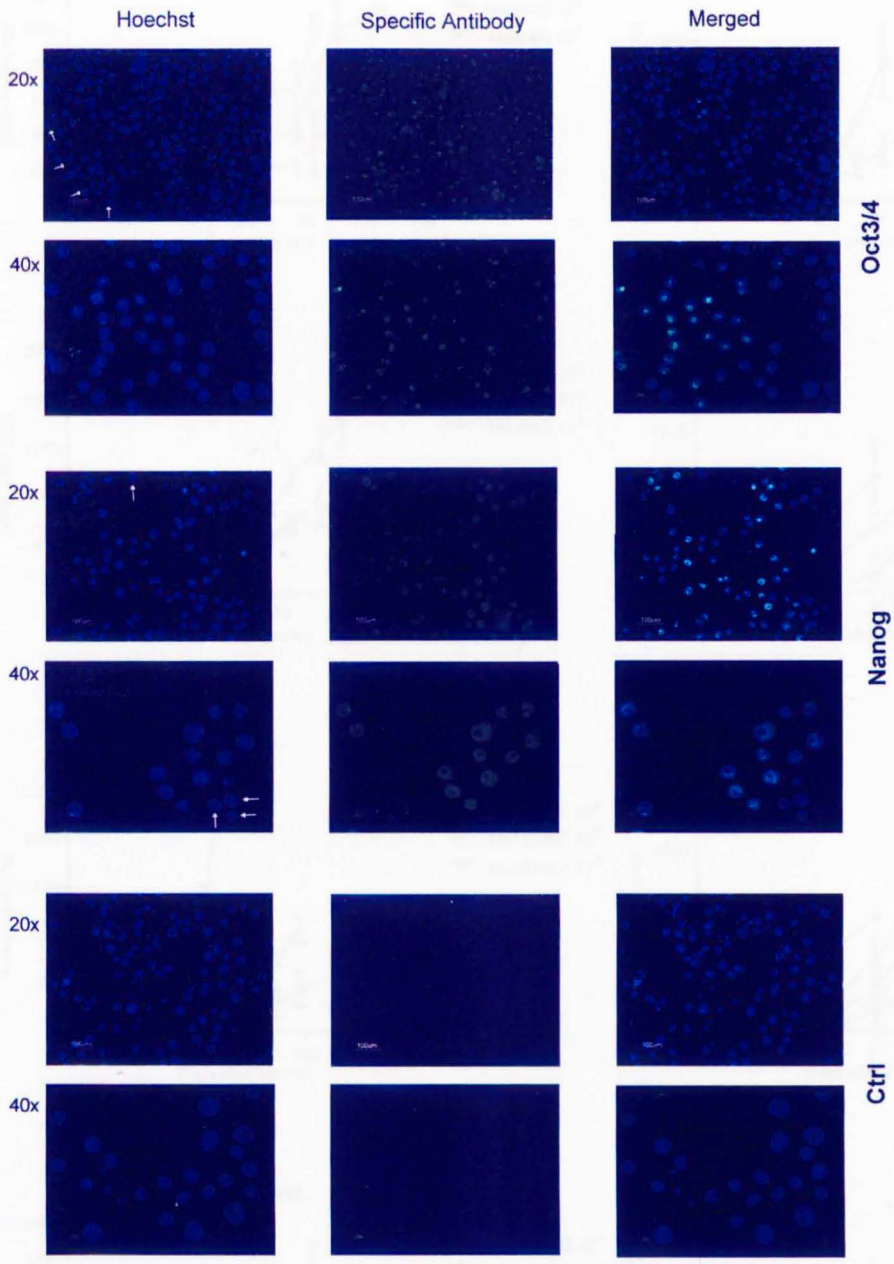
the examined markers. Staining only with the secondary antibody has been shown as control.

Figure 3S. Tumorigenic potential of melanospheres. Growth curves (GC) of primary (left) or serially transplanted (right) human melanoma xenografts generated by intradermal injection of different doses of melanosphere-derived cells. Tumor mass is expressed in mg and calculated as the mean ± SE of the masses of the growing tumors. For details on the number of mice treated with each inoculating dose, see Table 2.

Figure 4S. *Expression level of stem-related markers differs between human melanomas.* Immunohistological evaluation of the expression of the indicated stem-related markers in primary and paired metastatic melanomas. Reported images are representative of two independently analyzed tumor sections. Internal scale markers are indicated in each panel. The majority of tumor cells express (Pt3) CD166 and Nestin; rare CD133-positive cells could be found within the tumor mass in both primary and metastatic tumors. (Pt1) NGFR is heterogeneously expressed, and in some cases enhanced, in metastatic lesions. Images are representative examples of twelve primary and metastatic melanomas. Scale bar = 100 μm.

Figure 1


A
Me 204S
B
Osteocalcin
FABP4
Aggrecan
Melan-A/Mart-1
Me 204ADH
C
% of differentiated cells
100
80
60
40
20
0
Me 204S
Me 204ADH
Osteocalcin
FABP4
Aggrecan
Melan-A/MART-1


Hoechst
Specific Antibody
Merged
20x
40x
Oct3/4
20x
40x
Nanog
20x
40x
Ctrl

Primary engrafments

Serial engrafments

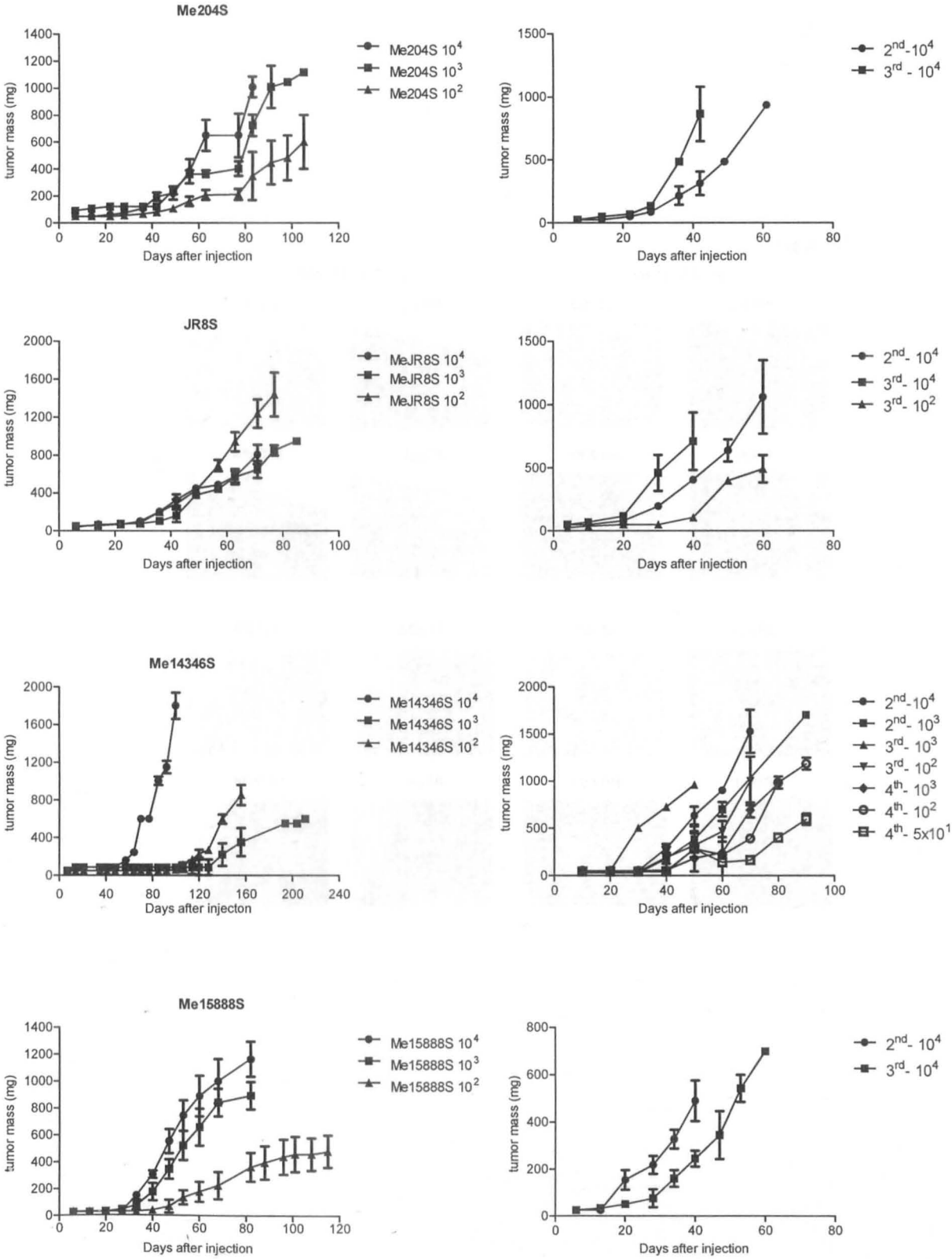
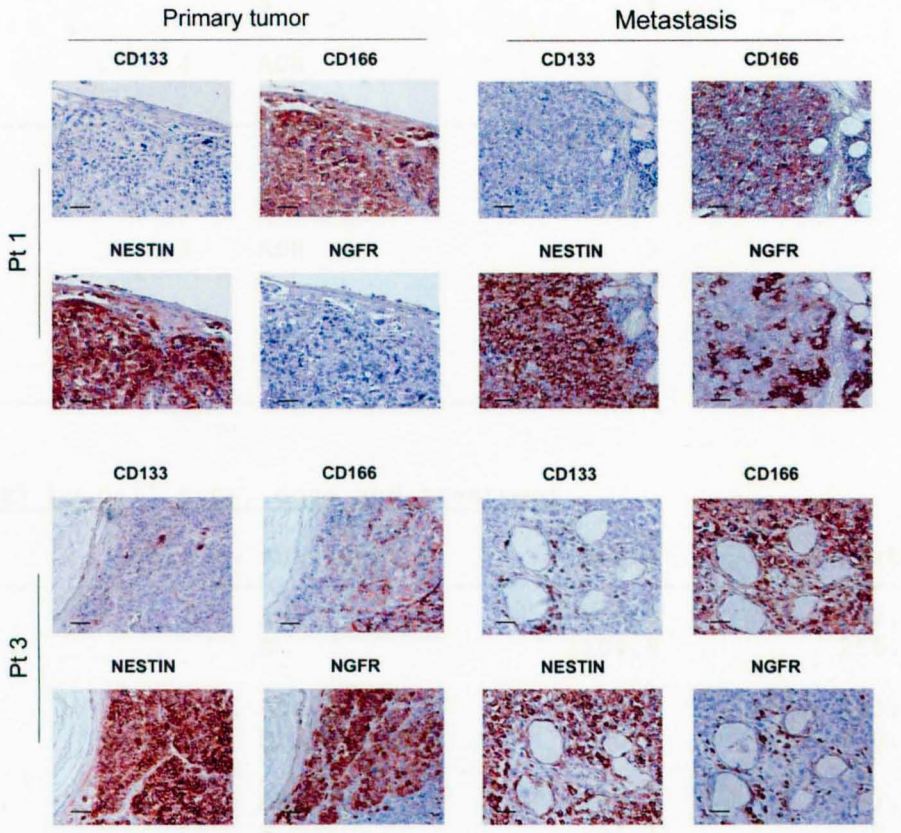


Figure S4


Primary tumor
Metastasis
CD133
CD166
CD133
CD166
Pt 1
NESTIN
NGFR
NESTIN
NGFR
CD133
CD166
CD133
CD166
Pt 3
NESTIN
NGFR
NESTIN
NGFR

Statistic Report

Number of tumors

Cell_type	dose	treatment	Somma
JR8	2	ADH	3
		S	5
	3	ADH	4
		S	5
	4	ADH	5
		S	5
T14346	2	ADH	2
		S	3
	3	ADH	3
		S	4
	4	ADH	5
		S	5
T204	2	ADH	0
		S	2
	3	ADH	5
		S	5
	4	ADH	5
		S	4

Mass (mean, SE) by cell type, dose and treatment

Cell_type	dose	treatment	Media	Err std
JR8	2	ADH	538.8	82.5
		S	1104.8	256.5
	3	ADH	363.8	110.7
		S	590.0	130.4
	4	ADH	257.9	67.1
		S	264.1	70.1
T14346	2	ADH	274.3	211.8
		S	418.2	182.3
	3	ADH	926.7	178.3
		S	523.9	170.7
	4	ADH	248.2	100.2
		S	643.5	160.8
T204	2	ADH	.	.
		S	602.5	197.5
	3	ADH	256.0	48.6
		S	869.3	150.4
	4	ADH	638.1	82.0
		S	1021.8	65.2

Analysis of tumor mass by cell type, dose and treatment

Dependent Variable: mass

Origine	DF	Type III SS	Media quadratica	Valore F	Pr > F
treatment	1	1082269.243	1082269.243	8.68	0.0045
Cell_type	2	474602.540	237301.270	1.90	0.1574
dose	2	265403.926	132701.963	1.06	0.3509

Parameter	Stima	Errore standard	Valore t	Pr > |t|
treatment ADH	-252.0495879	85.5393090	-2.95	0.0045

Least Squares Means

treatment	mass LSMEAN
ADH	450.087234
S	702.136822

Note:
P(treatment*Cell_type)=0.2948, P(treatment*dose)=0.7864

Analysis of tumor latency by cell type, dose and treatment

Type III Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
treatment	1	16.6024	<.0001
Cell_type	2	43.4657	<.0001
dose	2	73.2576	<.0001

Analysis of Parameter Estimates

Parameter		DF	Stima	Errore standard	95% Confidence Limits		Chi-quadrato	Pr > ChiSq
Intercept		1	3.0149	0.2007	2.6215	3.4084	225.55	<.0001
treatment	ADH	1	0.6242	0.1532	0.3240	0.9245	16.60	<.0001
	S (reference)							
Cell_type	JR8	1	-0.1803	0.1985	-0.5694	0.2088	0.82	0.3638
	T14346	1	0.9845	0.2084	0.5761	1.3929	22.32	<.0001
	T204 (reference)							
dose	2	1	1.8486	0.2187	1.4200	2.2771	71.47	<.0001
	3	1	0.7657	0.1714	0.4298	1.1016	19.96	<.0001
	4 (reference)							
Scale		1	0.6276	0.0570	0.5253	0.7499		
Weibull Shape		1	1.5933	0.1447	1.3335	1.9038		

Note:
P(Scale)<.0001, P(treatment*Cell_type)=0.4659, p(treatment*dose)=0.3564

Spheres of Influence in Cancer Stem Cell Biology

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TO THE EDITOR

We read with great interest the Commentary by Schatton and Frank (2010) that appeared in the July issue of *Journal of Investigative Dermatology*. They highlight one of the major findings of our article, published in the same issue of the Journal (Perego *et al.*, 2010): that malignant melanoma-initiating cells (MMIC) can also be found in melanoma cell lines when grown as adherent cells. We found that melanospheres could be derived from both melanoma single-cell suspensions obtained from fresh tumor specimens, and from melanoma cells previously adapted to grow as adherent cells.

However, it should be noted that we found the frequency of self-renewing cells was relatively low in adherent cells when compared with cells from melanospheres.

These *in vitro* quantitative differences were also reflected in the results from the *in vivo* tumorigenic assays using immunocompromised mice, clearly suggesting a greater enrichment of tumorigenic cells in melanospheres compared with adherent cells. Moreover the latency of xenografts generated in primary recipients by melanosphere cells was much shorter than that observed with adherent melanoma cells. Likewise, when xenografts were serially transplanted into secondary and tertiary hosts, the difference in successful tumor grafting between the two groups became even more apparent. Thus, for these reasons we believe the differences must reflect a biological difference in the two populations, although in our study the lack of a true limiting dilution analysis *in vivo* precluded any numerical quantification of MMIC in melanospheres and adherent melanoma cells, hence we cannot assess to what extent melanospheres are enriched in MMIC as compared with adherent melanoma cells.

On the other hand, it is accepted that spheres are not composed entirely of stem cells, as highlighted by two recent articles exploring heterogeneity in mammospheres and melanospheres (Pece *et al.*, 2010; Roesch *et al.*, 2010). Although we agree with Schatton and Frank, who advise caution in considering sphere formation as the definitive methodology for identifying cancer stem cells, these latest papers nevertheless stress the utility of sphere cultures to investigate cancer stem cell biology, including the fact that cancer stem cells may be more dynamically regulated than hitherto suspected (Heddleston *et al*, 2009; Vermeulen *et al.*, 2010)—and thus not strictly adhering to a hierarchical system. The considerable phenotypic heterogeneity that we found in our melanospheres can be ascribed to the relatively poor enrichment in melanoma stem cells, a view favored by Schatton and Frank, though equally well heterogeneity may be a reflection of MMIC diversity that increases with genetic instability as tumors progress (Piccirillo *et al*, 2009; Greaves 2010).

Regarding the prospective isolation of cancer stem cells from melanoma, a confusing array of markers have been proposed, ranging from none at all (Quintana *et al.*, 2008) to CD133 (Monzani *et al.*, 2007), ABCB5 (Schatton *et al.*, 2008), CD271 (Boiko *et al.*, 2010), and JARID1B (Roesch *et al.*, 2010). The emergence of genetically distinct clones with metastatic melanoma-initiating capacity may be behind this plethora of markers.

On the other hand, using the same methods (FACS isolation and sphere formation) has lead to the identification of a population of tumor-initiating cells expressing CD133 across a surprising range of tumor types, including colorectal cancer, Ewing's sarcoma, and hepatocellular carcinoma (O'Brien *et al.*, 2009). Thus, although these analytical approaches have limitations (Jensen and Parmar, 2006; Rosen and Jordan, 2009; Alexander *et al.*, 2009), they do seem useful for identifying tumor-initiating cells that are the same as cancer stem cells. Of note, one of our melanospheres, Me15888S, was highly enriched in cells expressing two cell-surface markers found to be associated with MMIC, namely CD133 and CD271 (Monzani *et al.*, 2007; Boiko *et al.*, 2010).

In conclusion, the field of cancer stem cells, especially in solid tumors, is still in its infancy. Several methodological issues still remain, most notably a careful assessment of the validity and limitations of the various analytical approaches. However, as highlighted by the authors of the Commentary, the 'must' that should always be kept in mind when evaluating any new data is indeed its relevance for patients and clinical transferability.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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